THE UNIVERSITY OF ALBERTA

REGULATION OF HUMAN AIRWAY EPITHELIAL CHLORIDE CHANNELS BY MATRIX METALLOPROTEINASES

by

YALI SHU

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirement for the degree of Master of Science.

DEPARTMENT OF PHYSIOLOGY

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DEGREE: Master of Science

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **REGULATION OF HUMAN AIRWAY EPITHELIAL CHLORIDE CHANNELS BY MATRIX METALLO-PROTEINASES** submitted by YALI SHU in partial fulfillment of the requirement for the degree of MASTER OF SCIENCE.

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ABSTRACT

The role of Matrix metalloproteinases (MMPs) in the regulation of Cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels was investigated. Gelatin-zymography and Western blot showed that two airway epithelial cell lines, Calu-3 and A549 secreted MMP-2 and MMP-9. Perforated whole-cell patch clamp studies showed that Cl^- current was affected by MMP inhibitor 1,10-phenanthroline in Calu-3 cells in which CFTR dominates Cl^- conductance, while Cl^- current was not affected in A549 cells in which non-CFTR dominates. Anti-MMP-2 antibodies increased whole-cell Cl^- current in Calu-3 cells but anti-MMP-9 antibodies did not. Inhibitory effects on Cl^- current were observed with human recombinant MMP-2. Single channel patch clamp showed that channels affected by phenanthroline in Calu-3 cells had similar biophysical properties to those of CFTR. Channel open probability was enhanced by phenanthroline but channel conductance was not. These results suggest that MMP-2 exert an inhibitory effect on CFTR Cl^- channels in Calu-3 cells.

To my parents Shu Qitai and Wang Dehua

for everything they have given me

and

To my husband Liu Shi and my lovely son Liu Shuyang,

for their love and support.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor, Dr. Marek Duszyk, for all he has taught me and shared with me. His academic guidance and understanding of personal matters have been invaluable. This work could not have been done without his great enthusiasm and generous assistance.

Special thanks to Drs. Constance Chik, Anthony K. Ho, Edward Karpinski, Malcolm King, and Redwan Moqbel, for their great concern of and precious advice on my work.

I would like to thank everyone in this research group and the department who have ever helped and encouraged me, especially Karen Thethi for improving my English and help with my course work; Bing Li for valuable suggestions on patch clamp; Dr. Greg Sawicki for Western blot study; Dan Liu and Lei Zhang for advice on cell culture, and Xiaobo Tang for interesting discussions on molecular biology.

My thanks also extend to the Department of Physiology General Office, for their administrative and secretarial support.

Finally I wish to thank my husband, Liu Shi, and my son, Liu Shuyang, whose love and patience I can always depend on. Without their support and encouragement, I would not have embarked on the journey for graduate studies.

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List of Abbreviations

ANOVA	analysis of variance
APMA	p-aminophenylmercuric acetate
ASL	airway surface liquid
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
CaCMKII	Ca^{2+} / calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cDNA	complement DNA
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ChTX	charybdotoxin
DIDS	4,4′-diisothiocyanatostilbene-2, 2-disulfonic acid
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DPC	diphenylamine-2-carboxylate
EDTA	ethylene-diaminetetra-acetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N', -tetraacetic acid
EME	Eagle's minimum essential medium
ENaC	epithelial sodium channel
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
MMPs	matrix metalloproteinases
mRNA	message RNA
MT- MMP	membrane type matrix metalloproteinase
Na^+ K^+ ATPase	Na^+-K^+ adenosine triphosphatase
NBD	Nucleotide-binding domain

ORCC	outward rectifying Cl^- channels
pH_i	intracellular pH
PKA	protein kinase A
PKC	protein kinase C
R domain	regulatory domain
rMMP-2	human recombinant MMP-2
SEM	standard error of the mean
SD	standard deviation
TEA	tetraethylammonium
TM	transmembrane domain
TIMPs	tissue inhibitor of metalloproteinases
TIMP-2	tissue inhibitor of metalloproteinase-2
TMD	transmembrane spanning domains
UTP	uridine triphosphate
4-AP	4-aminopyridine
$[Ca^{2+}]_i$	intracellular calcium concentration

Defined Symbols and units

I	total membrane current
V	applied voltage
V_m	membrane potential
E	reversal potential
P_o	open probability of channels
mV	unit of voltage: millivolts
pA	unit of current: picoamperes
pF	unit of capacitance: picofarads
pS	unit of conductance: picosiemens
MΩ	unit of resistance: megaohms
KHz	unit of frequency: kilohertz
g	gravitational acceleration: 9.81 m/s^2
°C	unit of temperature: degrees Celsius
$[X]_o$, $[X]_i$	extra and intracellular concentrations for ions
cell/ml	concentration; number of cells per milliliter
M, mM, μM, nM	concentration; molar, millimolar, micromolar, nanomolar
W/V	concentration: weight in grams per volume in liter
mg/ml	concentration: weight in milligrams per volume in milliliters
mm, nm	unit of length: millimeters, nanometers
ms, s, min	unit of time: milliseconds, seconds, minutes

Chapter 1 Introduction

One of the pulmonary defense mechanisms that helps prevent infection and damage from inhaled particles involves the transport of these particles from the airways by airway surface liquid (ASL). The quantity and composition of this fluid is controlled, in part, by the transport of ions through the airway epithelium. Transepithelial ion fluxes can produce a net secretory flux of chloride from submucosa to mucosa or a net absorptive flux of sodium in the opposite direction. Abnormal transepithelial ion transport has been associated with several pulmonary diseases, particularly cystic fibrosis (CF).

Movements of ions across epithelia are determined by transport mechanisms in three different membranes: apical, basolateral and junctional. These mechanisms comprise ionic permeation through channels, active transport and electrically neutral cotransport of ions. Since electroneutrality must be preserved, the transport of each kind of ion is always coupled to the transport of other ions. Current evidence indicates that airway epithelial Cl^- channels play a crucial role in the homeostasis of ASL and studies of their regulatory mechanisms are a subject of this thesis.

1.1 Airway mucociliary clearance and ion transport

Airway surface liquid is a thin layer of fluid lining the pulmonary airway surface. It is a heterogeneous, viscoelastic fluid containing water, carbohydrates, proteins, lipids and electrolytes (King & Rubin, 1994). ASL is a product of the respiratory system and is of primary importance in lung defense. A closer examination of ASL indicates that it can be

divided into two relatively distinct layers: a superficial mucous (gel) and a periciliary fluid (sol) layer (Yoneda, 1976). The presence of the two liquid layers is believed to be critical for effective mucociliary clearance, the process that keeps the airway surface clean. The cilia are able to beat in the low viscosity sol layer and their tips contact the underside of the mucous blanket, driving it and any entrapped dirt, chemicals, microorganisms to the mouth where it is expectorated or swallowed. The clearance of entrapped materials and microorganisms in mucus involves efficient ciliary stroke mechanics, which are dependent on periciliary liquid depth being approximately equal to the cilial height. Too deep a periciliary layer would prevent the cilia from contacting the gel layer and therefore prevent the movement, whereas too shallow a layer would prevent the efficient ciliary power- and recovery- stroke. The effectiveness of the mucociliary clearance also depends on the viscoelasticity of the mucus gel which is partially decided by the hydration of the mucus gel (Widdicombe, 1997).

It is generally accepted that ion transport through the airway epithelium is responsible for maintaining the correct composition and volume of the airway surface liquid. In all airway epithelia studied so far, active Na^+ absorption, active Cl^- secretion, or a combination of the two, contributes almost entirely to the ion transport activities in epithelial cells (Widdicombe et al., 1993). Active secretion of Cl^- will make the airway lumen electrically negative, inducing net passive movement of Na^+ into the lumen, largely through the tight junctions. The resulting transfer of salt from the serosal to mucosal surface of the epithelium creates an osmotic gradient drawing water into the lumen. Similar arguments show that active absorption of Na^+ should promote absorption of fluid from lumen to blood. These fluid movements, by altering the depth of the

periciliary sol layer and/or the hydration of the mucous gel, will affect the efficiency of mucociliary clearance (Sherif & Boucher, 1997). The most convincing evidence for a role of active transepithelial ion transport processes in regulation of mucus hydration and mucociliary clearance comes from studies on cystic fibrosis (CF), a genetic disease caused by mutations of cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes the CFTR Cl^- channel (Knowles, et al., 1983). The characteristic accumulation of airway mucous secretions in CF has been linked to decreased airway Cl^- secretion (Widdicombe, et al., 1985) as well as increased Na^+ absorption (Boucher, et al., 1986).

The cellular mechanisms for active Na^+ absorption and active Cl^- secretion are shown in Figure 1-1. Active absorption of Na^+ by airway epithelia follows the basic mechanism proposed 40 years ago by Koefoed-Johnsen and Ussing for frog skin (Koefoed-Johnsen & Ussing, 1958). Sodium ions cross the apical membrane via Na^+ channels down both chemical and electrical gradients. The accumulated intracellular Na^+ is then extruded across the basolateral membrane by the Na^+-K^+ adenosine triphosphatase (Na^+-K^+ ATPase, or Na^+-K^+ ATP pump) restricted to this membrane. To maintain the electroneutrality, Cl^- follows the Na^+ from lumen to blood, largely through the paracellular pathway. Chloride enters the basolateral membrane via an electrically neutral Na^+-K^+-$2Cl^-$ cotransport system in which Na^+ enters the cell down a favorable electrochemical gradient, providing energy to drive Cl^- against its electrochemical gradient (Frizzell et al., 1979). Chloride ions accumulate within the secretory cell above their electrochemical gradient and exit passively down this gradient through

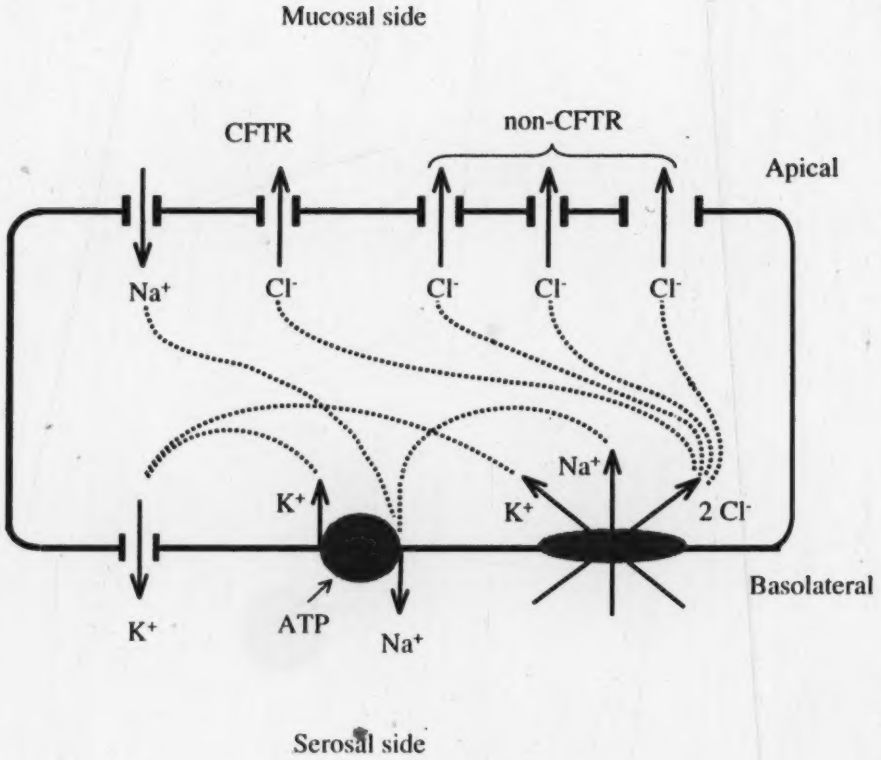

Figure 1-1. A model of ion transport in an airway epithelial cell

an apical membrane Cl^- channel. The increased intracellular K^+, which is pumped in by the Na^+-K^+ ATPase and transported in by the Na^+-K^+-$2Cl^-$ cotransport, exits the cell through basolateral membrane K^+ channels (Welsh, 1987).

It was observed that under basal conditions, there is no Cl^- secretion in human airway epithelia since the Cl^- ions are close to electrochemical equilibrium across the apical membrane, and thus little net Cl^- ion flow occurs (Willumsen et al., 1989a). However, under certain circumstances the same cells can be stimulated to secrete Cl^-. For example, hyperpolarization of the apical membrane via the blockade of the apical membrane Na^+ conductance causes Cl^- to rise above its electrochemical equilibrium and results in secretion of Cl^- via apically located Cl^- channels (Willumsen et al., 1989a).

1.2 Ion channels and their regulation in airway epithelia

Transepithelial ion transport requires the presence of different transport elements, including ion channels, in both apical and basolateral membranes. A discussion of ion channels requires an understanding of the characteristics of individual channels as well as an indication of how these channels contribute to net current. The most distinctive characteristic for ion channels is the channel conductance. By definition, conductance is the inverse of resistance measured via Ohm's law. Single-channel conductance is a unique characteristic of a channel, whereas a net conductance is an approximate measurement of how many channels are open and thus are contributing to the net current. Analysis of ion channels has been enhanced with the advent of patch clamp technique, which characterizes ion channels by conductance, selectivity, opening and closing

kinetics, and inhibition by blockers (Hamill et al., 1981). Our understanding of the structure and function of ion channels is also extended by the purification and molecular cloning of channel proteins. The ion channels found in airway epithelial cells include Na^+, K^+, Cl^- and the less well known Ca^{2+} channels. These ion channels have a range of biophysical properties and are regulated by a variety of cellular mechanisms. In this section, I will focus on Na^+, K^+, and Cl^- channels since they are the crucial components in the airway epithelial ion transport system.

1.2.1 Epithelial sodium channels

In all absorptive epithelia studies so far, sodium channels have been found located exclusively in the apical membranes (Garty & Palmer, 1997). These channels display high selectivity for Na^+ over K^+ ions and are blocked by 3,5-diamino-N-(aminoiminomethyl) –6-chloropyrazinecarboxamide, commonly known as amiloride. Unlike Na^+ channels of excitable tissues, the epithelial Na^+ channels are insensitive to voltage and to inhibition by toxins such as tetrodotoxin. The single-channel conductance of these Na^+ channels has been reported to range from 3 to 25 picosiemens (pS) (Garty & Palmer, 1997). In general, the Na^+ /K^+ selectivity of these channels decreases with the increasing channel conductance (Hamilton KL et al., 1985; Garty & Palmer, 1997).

Several major experimental strategies have been employed to clone the epithelial sodium channel. The classic approach of protein purification followed by amino acid sequencing of a peptide has been hampered by the lack of a rich source of channel protein. Most of the epithelial channels are present in a few copies (10^2 – 10^3) per cell, so it becomes very difficult to purify the desired protein in sufficient amounts for

sequencing. In spite of the difficulty, Benos et al. (1986, 1987) used an amiloride-affinity chromatography and were able to isolate a protein complex of ~700 KDa from bovine kidney and A6 cells, which upon reduction was resolved into five major polypeptide bands with apparent molecular masses of about 300, 150, 95, 70, and 55 KDa. The radioactively labeled amiloride analog ^{3}H-methylbromoamiloride, was found to bind covalently to the 150 KDa subunit only. When the 150 KDa subunit was reconstituted in lipid bilayers, an amiloride-sensitive current was observed with a single-channel conductance of about 9-60 pS and high selectivity for Na^+ over K^+ ions (Matalon et al., 1996). Although the small amount of protein recovered and the multimeric nature of the complex did not allowed the peptide sequencing to be done, these studies suggested that only one subunit was required to form ion selective channels after reconstitution into lipid bilayers. Several other groups have also attempted to use this strategy to clone a sodium channel. However, because amiloride can bind to many other membrane transporters and intracellular proteins, the isolation methods that rely on amiloride affinity were shown to be nonspecific for the sodium channel (Chassande et al., 1994).

Another strategy used to clone the epithelial sodium channel, which turned out to be much more successful, was expression cloning in *Xenopus* oocytes. This method was especially suitable for cloning epithelial sodium channels, since oocytes do not express any endogenous amiloride-sensitive conductances. Using this method, Canessa et al. (1993, 1994) have cloned a novel epithelial Na^+ channel (ENaC), which was shown to consist of three homologous subunits α, β and γ, with molecular masses of 72, 78 and 75 KDa, respectively. Each subunit was shown to contain two putative transmembrane domains, relatively short cytoplasmic NH_2^+ and $COOH^-$ terminals, and a large

extracellular loop. When all ENaC cDNAs were coexpressed in Xenopus oocytes, a typical epithelial Na^+ current was recorded. The channel exhibited a high selectivity for Na^+ versus K^+ with a single-channel conductance of 4.6 pS and could be blocked by amiloride (K_i =104 nM). No substantial voltage dependence was observed in these Na^+ channels.

The regulation of airway epithelial Na^+ channels contributes to the control of the volume of airway surface liquid (Noone, et al., 1994). Mineralocorticoids might be expected to play a role in Na^+ transport regulation in airway epithelia, like they do in renal epithelia (Duchatelle et al., 1992), but in vitro and in vivo studies failed to demonstrate any effect of these hormones on airway Na^+ conductance (Boucher & Gatzy, 1983; Knowles et al., 1985). The agonists bradykinin and adenosine triphosphate (ATP) have been reported to increase Na^+ transport rates in human nasal epithelia (Mason et al., 1991; Clarke et al., 1992a). Intracellular pH (pH_i) also influences human apical Na^+ transport, with inhibition occurring at low pH_i ~6.8 and activation at high pH_i ~ 7.8 (Willumsen et al., 1992). Agonists and agents such as epinephrine that enhance intracellular adenosine 3', 5'-cyclic monophosphate (cAMP) levels increase the open probability of these channels (Boucher et al., 1986). Recently, Ca^{2+} was reported to produce a voltage-dependent block, affecting open probability but not the unitary conductance of rENaC and the block could be released by mechanical stimulation (Ismailov et al., 1997). Furthermore, regulation of epithelia Na^+ channel by CFTR, was recently reported (see 1.2.3.1) (Ismailov et al., 1996).

1.2.2 Epithelial potassium channels

Potassium channels present in the basolateral membrane of respiratory epithelial cells play an important role in maintaining the membrane potential and driving force for Cl^- secretion (McCann et al., 1990a). Three major types of the epithelial K^+ channels have been reported in airway epithelia. They are voltage-dependent K^+ channels, Ca^{2+}-dependent K^+ channels and stretch-activated K^+ channels. There are no molecular biological data available for these airway epithelial K^+ channels so far, but their biophysical and pharmacological properties have been well characterized.

A voltage-dependent K^+ channel with distinct properties has been identified in airway epithelia (DeCoursey, 1988). This K^+ channel activates with a sigmoid time course upon membrane depolarization, and inactivates during maintained depolarization. The average maximum whole-cell K^+ conductance was 1.6 nS. External tetraethylammonium (TEA), 4-aminopyridine (4-AP) and charybdotoxin (ChTX) can block this voltage-dependent K^+ current (DeCoursey, 1995). These biophysical and pharmacological characteristics suggest that the airway epithelial voltage-dependent K^+ channel is similar to the delayed rectifier reported in neuronal tissues.

Two types of Ca^{2+}-dependent K^+ channels have been reported in airway epithelia. The first type, the Ca^{2+}-dependent inwardly rectifying K^+ channel is activated only by cytosolic Ca^{2+} and is insensitive to membrane potential. The single-channel conductance for this Ca^{2+} -dependent K^+ channel was reported to be around 35 pS. The active Ca^{2+}-dependent inwardly rectifying K^+ channel was shown to be sensitive to extracellular TEA and ChTX, and intracellular 4-AP, quinine, ATP and Ba^{2+} (Roch et al., 1995). The

cAMP-mediated pathway was shown to regulate the activity of this type of K^+ channels by either directly reacting with the channel protein or indirectly enhancing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Loussouarn, 1996; McCann & Welsh, 1990b). The physiological function of this Ca^{2+} -dependent K^+ channel was believed to be related directly to the Cl^- secretion and thus volume regulation (Sheppard & Welsh, 1993).

Another type of Ca^{2+} -dependent epithelial K^+ channel, termed maxi K^+ channel, was reported in airway epithelia (Kunzelmann, 1989a; Ridge et al., 1997). This channel showed burst appearance with rapid flickering within the burst. The single-channel conductance was between 150-300 pS. The channel open probability was only slightly voltage dependent, whereas an increase in intracellular Ca^{2+} concentration led to a marked increase in the channel open probability (Kunzelmann, 1989b). Blockade by Ba^{2+}, quinine and TEA was reported from both sides, whereas ATP inhibited the maxi K^+ channel from only the cytosolic side.

A K^+-selective stretch-activated channel was recently reported (Kim et al., 1993). This channel had a single-channel conductance of 65pS. The open probability of this stretch-activated K^+ channel was increased from < 0.01 to 0.30 by 50 mmHg of suction. The biophysical properties of this channel were found to be distinct from those of the other types of well-characterized basolateral K^+ channels.

As described above, the K^+ channel activity can be regulated by voltage, intracellular Ca^{2+} concentration, and mechanical factors. The activity of K^+ channels can also be regulated through PKA and PKC dependent phosphorylations. A recent report indicates that extracellular ATP stimulates K^+ secretion across cultured human airway epithelium (Clarke et al., 1997). The accelerated secretion of K^+ hyperpolarizes the cell

membrane thus enhances Cl^- secretion (Clarke et al., 1997). CFTR is also reported to regulate volume-activated K^+ channels (Valverde et al., 1995).

1.2.3 Epithelial chloride channels

Airway epithelial Cl^- channels play a fundamental role in maintaining airway surface fluid homeostasis. They have received intense attention because of their important role in cystic fibrosis. Understanding these Cl^- channels will enable the development of CF treatment. There are at least four types of Cl^- conductances located in the apical membrane of airway epithelial cells. They are illustrated in figure 1-2 along with their different regulatory mechanisms. Of all Cl^- channels analysis, only CFTR and ClC have been cloned, the molecular structures of other Cl^- channels are little known and need further studies to elucidate.

1.2.3.1 cAMP-mediated Cl^- channels

The evidence that Cl^- channels are defective in cystic fibrosis has stimulated a great interest in identification of Cl^- channels (Quinton, 1983). Cloning of the CF gene was accomplished by series of experiments involving saturation mapping as well as chromosome walking and jumping techniques (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989). Subsequent studies showed that this gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), was a cAMP-mediated Cl^- channel (Anderson & Welsh, 1991a; Tabcharani 1991; Bear et al., 1992). Expression of CFTR cDNA in heterogeneous cells with little or no expression of endogenous CFTR,

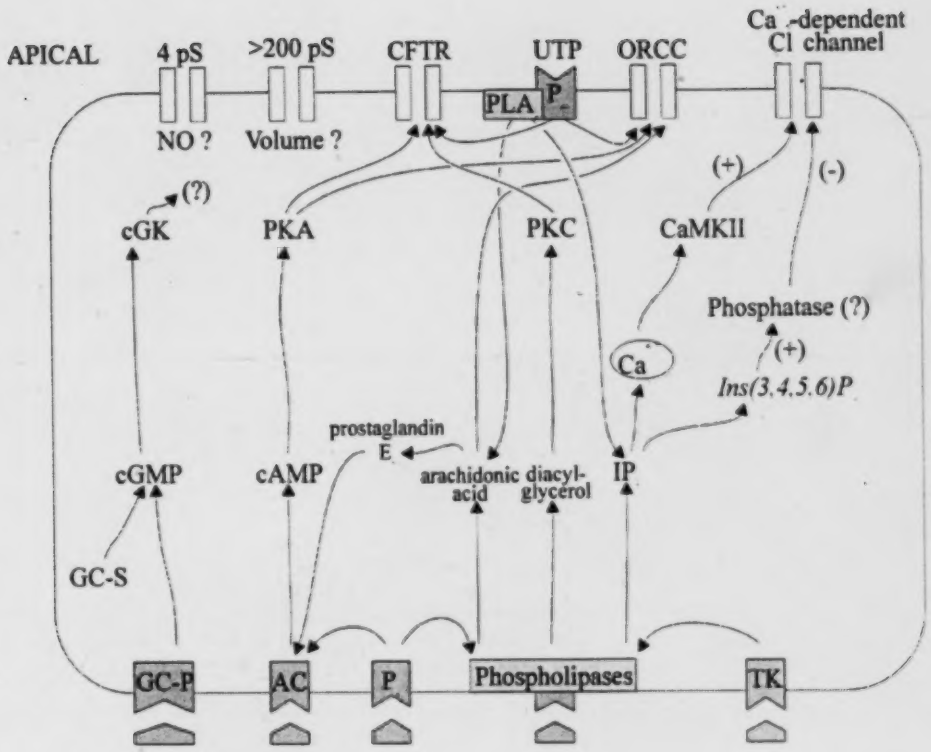

Figure 1-2. Illustration of different types of Cl- conductances located in the apical membrane of an airway epithelial cell along with proposed regulatory pathways of these channels. P1, 2: purinergic receptors; AC: adenylyl cyclase; TK: tyrosine kinase; DAG: diacylglycerol; GC: guanylyl cyclase; CaMKII: type II Ca/calmodulin dependent kinase; AA: arachidonic acid; PGE2: prostaglandin E2; PLC, A2: phospholipases C and A2.

resulted in a cAMP-induced linear voltage-current relationship, with no obvious voltage-dependent or time-dependent activation or inactivation (Anderson et al., 1991b; Dalemans et al., 1991). The single-channel conductance is about 8-10 pS, with a halide permeability sequence $Br^- > Cl^- > I^-$. The channel can be blocked by diphenylamine-2-carboxylate (DPC) but can not be blocked by the stilbene derivatives, such as 4,4′-diisothiocyanatostilbene-2, 2-disulfonic acid (DIDS) (Anderson et al., 1991b, Dalemans et al., 1991). The recombinant CFTR showed similar properties to those of endogenously expressed or purified and reconstituted CFTR (Tabcharani et al., 1990; Bear et al., 1992).

CFTR protein consists of twelve transmembrane spanning domains (TMD), two nucleotide-binding domains (NBD), and a novel regulatory domain (R domain) that contains several consensus sites for protein kinase A- (PKA-) and protein kinase C- (PKC-) mediated phosphorylation (Figure 1-3) (Riordan et al., 1989). CFTR is also called "ABC" transporter, so called because it belongs to a superfamily of membrane proteins containing a consensus ATP Binding Cassette (Hyde et al., 1990). The location of CFTR has been reported in many transporting epithelia. In the lung, CFTR has been localized primarily to the apical membrane of epithelial cells in submucosal glands of the proximal airways (Engelhardt et al., 1992) and is also expressed in the distal airways and the alveolar region (Engelhardt et al., 1994).

The regulation of CFTR activities includes the phosphorylation of its R domain by PKA or PKC and the interaction of its NBD with ATP. Activation of CFTR minimally requires PKA phosphorylation on its R domain as well as binding of ATP to both NBDs

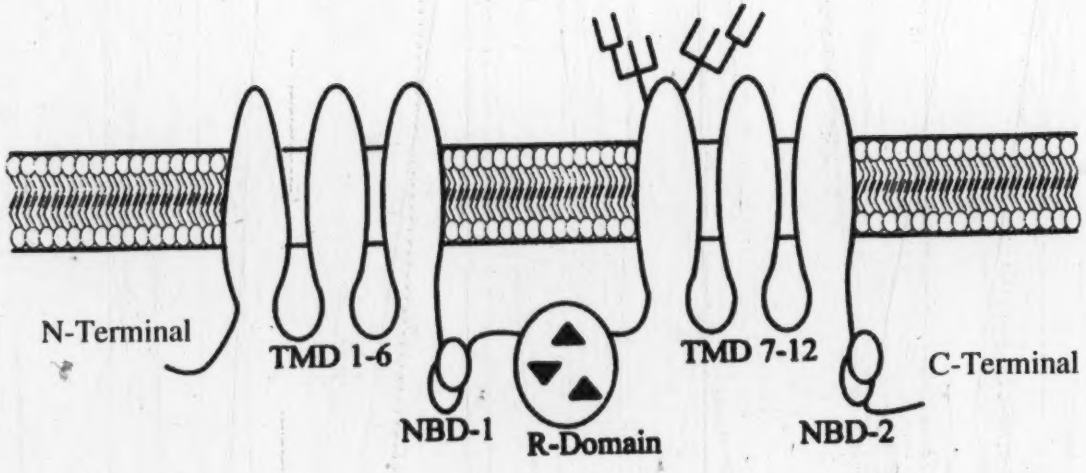

Figure 1-3. Schematic representation of the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR appears as a "pseudo-homodimer," with each half of the molecule consisting of six transmembrane domains (TMD) and a nucleotide-binding domain (NBD). The two halves are joined by a novel "regulatory" domain (R-domain) that contains consensus PKA-phosphorylation sites (▲) and consensus PKC-phosphorylation sites (▼) (adapted from Sherif and Boucher, 1997).

and subsequently hydrolysis of ATP (Tabcharani et al., 1991; Anderson et al., 1991c; Cheng et al., 1991, Foskett, 1998). There are ten PKA phosphorylation sites on the R domain, but none of them were shown to be essential (Chang et al., 1993). In fact, it was shown that protein kinase A (PKA) still activated CFTR chloride channel after mutagenesis of all 10 PKA consensus phosphorylation sites. Another recent paper showed the activation of CFTR without the phosphorylation of any of these ten sites (Weinreich et al., 1997). These results indicate that there may be some more PKA consensus phosphorylation sites other than the ten known high-affinity consensus sequences. Besides PKA, consensus sequences for PKC were also identified on the R domain (Riordan et al., 1989) and the PKC phosphorylation showed a synergistic action with PKA on CFTR activation (Tabcharani et al., 1991; Jia et al., 1997).

In addition to being a cAMP-mediated Cl^- channel, CFTR also functions as a regulator of the activities of other apical ion channels including amiloride-sensitive Na^+ channels, outwardly rectifying Cl^- channels (ORCC) and epithelial K^+ channels (Jentsch, 1996). Egan (1992) reported that CFTR activates another type of cAMP-activated Cl^- channels, ORCC. This regulation is achieved through ATP, which leaves the cell in response to CFTR stimulation (Jovov et al., 1995). Extracellular ATP then activates ORCC by interacting with purinergic receptors (Stutts et al., 1994; Schwiebert et al., 1995). Whether direct interaction, intracellular Ca^{2+}, and / or other pathways mediate this activation is not yet clear. The mechanism of ATP exiting the cell in response to CFTR remains controversial (Devidas & Guggino, 1997). One hypothesis suggested that CFTR itself could transport ATP and therefore function as a dual ATP and chloride channel (Reisin et al., 1994; Abraham et al., 1997). Another hypothesis suggests that CFTR might

regulate ATP conductance, based on the evidence that no direct ATP transportation by CFTR was observed (Reddy et al., 1996; Grygorczyk et al., 1996).

Recent data showed that CFTR led to cAMP-stimulated inhibition of co-transfected epithelial Na^+ channels (Stutts et al., 1995, 1997). Another report showed that CFTR could directly down-regulate single Na^+ channel activity by co-incorporating both channels into planar lipid bilayers (Ismailov et al., 1996). These results explain the observation that airway epithelia of cystic fibrosis patients show an increase in Na^+ currents. However, the molecular mechanism underlying this interaction between both channel molecules is still unclear. One study showed that actin may play a role in the regulation of epithelial Na^+ channel by CFTR (Ismailov et al., 1997). Besides apical membrane Na^+ channels and ORCC, volume-activated K^+ channels are also affected by CFTR (Valverde et al., 1995).

It is now widely accepted that the CFTR Cl^- channel is the predominant cAMP-regulated Cl^- channel in the apical membrane of epithelial cells and that the genetic defects in the activity of this channel resulting from CF gene mutation are the underlying cause of cystic fibrosis.

Cystic fibrosis is the most common fetal autosomal-recessive genetic disorder of the Caucasian population. In normal human epithelia, CFTR is located in the apical membrane, where it mediates cAMP-regulated transepithelial Cl^- transport. In CF epithelia, CFTR is missing from the apical membrane or can not function normally (Welsh & Smith, 1993). This defect causes a loss of cAMP-dependent Cl^- secretion. The disease reflects a disturbance of the homeostasis of airway surface liquid resulting from abnormal epithelial transport: decreased Cl^- secretion and increased Na^+ absorption. The

altered volume and composition of the airway surface liquid impairs the mucociliary clearance, which causes chronic infection of airways.

The CF gene, responsible for this disease, has been identified on human chromosome 7 (Tsui et al., 1985; Wainwright et al., 1985; White et al., 1985), and subsequently cloned (Riordan et al., 1989; Rommens et al., 1989). It is 325 kilobase pairs (Kb) long with 24 exons coding for 1480 amino acids of CFTR protein. More than 400 mutations have been localized on the CF gene since its discovery. The most frequent mutant form of CFTR, $\triangle$F508, caused by a single deletion of a phenylalanine residue at position 508, represents about 70% of total mutations of CF. This mutation occurs in the first nucleotide-binding domain (NBD1) and was thus speculated to affect the mechanics of Cl^- transport. Patients with homozygous for the $\triangle$F508 mutation often suffer from the most severe symptoms, including pancreatic insufficiency, meconium ileus, and chronic airways infection.

Another group of the cAMP-mediated Cl^- channels that has been reported in airway epithelia is the outwardly rectifying Cl^- channels (ORCC) (Welsh & Liedtke, 1986; Anderson et al., 1992). This channel is so called because it conducts more current at depolarizing potentials than at normal physiological potentials and is therefore rectifying. Unlike CFTR, the single-channel recording of ORCC shows a nonlinear current-voltage relationship, with a conductance at zero membrane potential of approximately 30 pS and a sensitivity to the blockade of stilbene derivatives (Ward et al., 1991). In addition to these differences, ORCC was reported to be voltage-dependent, with channel activation occurring at strong depolarizing voltages (>50 mV) for seconds to minutes (Welsh et al.,

1989). These differences in their biophysical properties and blocker sensitivities allow a clear separation of CFTR and ORCC.

The regulation of ORCC includes PKA- and PKC- dependent phosphorylation, binding of ligands to purinergic receptors, and CFTR. Welsh et al (1989) demonstrated ORCC to be activated by PKA- and PKC-dependent phosphorylation. However, the effect of PKC on ORCC was shown to be Ca^{2+}-dependent with reduced PKC activation at $[Ca^{2+}]_i$ > 150 nanomolar (nM) (Li et al., 1989). Binding of purinergic receptors by ligands such as ATP or uridine triphosphate (UTP) activates ORCC. Recent studies show that CFTR regulates ORCC activities by influencing ATP transport (Devidas et al., 1997).

1.2.3.2 Ca^{2+}-mediated Cl^- channels

The apical Ca^{2+}-mediated Cl^- channel is not as well known as CFTR or ORCC. In the CFTR knockout (-/-) mouse, the airways remain relatively normal despite the absence of CFTR and cAMP-mediated Cl^- secretion (Snouwaert et al., 1992; Clarke et al., 1992b). Subsequent studies showed that an alternate Cl^- channel regulated by $[Ca^{2+}]_i$ may compensate for the absence of CFTR (Clarke et al., 1994). The finding of an alternate Cl^- conductance has stimulated interest in identifying the single channel properties that correspond to this current.

Unlike current recorded from CFTR, whole-cell Ca^{2+}-dependent Cl^- current shows strong outward rectification, with time-dependent activation at depolarizing potentials (Cliff & Frizzell 1990; Anderson & Welsh 1991a). Similar to ORCC, the Ca^{2+}-dependent Cl^- current was shown to be sensitive to DIDS blockade, and has higher permeability to I^-

than to Cl^-. The mechanism of the activation of Cl^- current by Ca^{2+} was demonstrated to be by multifunctional Ca^{2+} / calmodulin-dependent protein kinase II (CaCMKII) phosphorylation, not by PKC (Wagner et al., 1991; Fuller et al., 1994).

The molecular structure of this Ca^{2+}-dependent Cl^- channel is little known to date. However a cAMP insensitive Cl^- channel was recently purified from bovine trachea through immunoaffinity purification chromotography (Fuller et al., 1994). This channel has a molecular weight of 140 KDa. When incorporated into lipid bilayers, it showed insensitivity to PKA, but was activated by raised intracellular Ca^{2+} concentration to 3 μM or above. The measured single-channel conductance of this channel ranged from 25-30 pS, with a linear current-voltage relationship under symmetrical Cl^- conditions. Similar to the recorded Ca^{2+}-mediated Cl^- channel before, the 140 KDa Cl^- channel was also sensitive to DIDS blockade. This Cl^- channel was also shown to be activated via Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation (Cunningham et al., 1995). Interestingly, recent studies have shown that Ca^{2+}-activated chloride channels may also be involved in mediating cell-to-cell adhesion (Elble et al., 1997).

1.2.3.3 ClC Cl^- channels

The ClC Cl^- channels form a large gene family of homologous proteins. Similar to CFTR, defective ClC Cl^- channels are also linked to various diseases such as myotonia and Dent's disease (Jentsch, 1996). Up to date, eleven ClC Cl^- channels have been cloned from various tissues and their expression pattern and proposed functions are listed in Table 1-1. Each channel protein is composed of about 700-900 amino acids and the

Table 1-1. Expression and functions of ClC Cl^- channels

Channel	Expression	Proposed functions	References
ClC-0	Electric organ	Stabilization of V_m	Jentsch et al., 1990
ClC -1	Skeletal muscle	Stabilization of V_m	Steinmeyer et al., 1991
ClC -2	Ubiquitous (including epithelial tissues)	Volume regulation, Limiting $[Cl^-]_i$	Thiemann et al., 1992
ClC -2G	Gastric mucosa	HCl secretion	Malinowska et al., 1995
ClC -3	Brain, kidney	Memory formation	Kawasaki et al., 1994
ClC -4	Skeletal muscle, Kidney, Brain	?	Van Slegtenhorst et al., 1994
ClC -5	Kidney, Brain	Urine formation	Steinmeyer et al., 1995 Sakamoto et al., 1996
ClC -6	Ubiquitous	?	Brandt & Jentsch 1995
ClC -7	Ubiquitous	?	Brandt & Jentsch 1995
ClC -K_1	Kidney	Urine concentration	Uchida et al., 1993
ClC -K_2	Kidney	Urine concentration	Adachi et al., 1994

V_m: membrane potential; $[Cl^-]_i$ intracellular Cl^- concentration; HCl: hydrochloric acid

molecular mass ranges from 70-110 KDa. Based on the homology of the channel proteins, these ClC channels can be assigned to one of three branches. Members of the first branch, including ClC-0, ClC-1, ClC-2, ClC-2G, ClC-K1, and ClC-K2, share 40-80 % amino acid identities. The second branch, which consists of ClC-3, ClC -4, ClC-5 shows about 80% homology to each other. The last branch includes two putative channels ClC-6, ClC-7, which have about 45% amino acid identities to each other and only have about 20-30% homology with the members of the other two branches (Foskett, 1998).

All members of the ClC family share a common topology, consisting of twelve transmembrane domains (TM1-TM12), a conserved cytoplasmic region D_3, and cytosolic N- and C- terminals. Analysis of new ClC genes suggests that the TM4 domain does not traverse the plasma membrane and most likely forms an extracellular loop between TM3 and TM5 domains (Jentsch, 1996).

The biophysical and pharmacological properties of these ClC channels are summarized in Table 1-2. Despite the similarities in their overall topology and amino acid sequences, each member of the ClC family exhibits different biophysical properties, blocker sensitivities and regulatory mechanisms. Of all these ClC family members, only the ClC-2 Cl^- channel was shown to have a wide tissue distribution and be highly expressed in most epithelial tissues, including the lung. The physiological role of this channel is not yet clearly defined. Its proposed functions include cell volume regulation and limiting intracellular Cl^- concentration (Jentsch, 1996).

Table 1-2. Electrophysiological properties of the recombinant ClC Cl^- channels

Channel	Rectification & anion selectivity	Voltage Dependence	Single-Channel Conductance (pS)	Blocker Sensitivity	Regulation
ClC -0	Linear $Cl^->Br^->I^-$	Fast gating at $+ V_m$ Slow gating at $- V_m$	~ 10	DPC, 9-AC, DIDS	Voltage
ClC -1	Inward $Cl^->Br^->I^-$	Open at $+ V_m$	~ 1	9-AC, DPC	Voltage
ClC -2	Inward $Cl^->Br^->I^-$	Open at $- V_m$	~ 3-5	9-AC, DPC	Swelling Voltage
ClC -2G	Linear $I^->Cl^->NO_3^-$	Open at $+ V_m$	~ 7	?	PKA, pH
ClC -3	Outward $I^- > Cl^- = Br^-$	Voltage independent	?	DIDS	PKC, CaCMK
ClC -5	Outward $I^- > Cl^- > F^-$	Open at $+ V_m$	?	DIDS, DPC	Voltage
ClC -K_1	Outward $Br^- > Cl^- > I^-$	Voltage independent	?	DIDS, 9-AC	pH, Ca^{2+}
ClC -K_2	Outward $Br^- > Cl^- > I^-$	Open at all V_m	?	DIDS	voltage

1.2.3.4 Stretch-activated Cl^- channels

Mechanical stimulation to cells is another important physiological factor for regulating epithelia Cl^- secretion. At least three types of stretch-activated Cl^- channels were reported. The first one, ClC-2 chloride channel, shows both voltage- and swelling stretch-activated properties as described earlier. Another putative cell-swelling stretch-induced Cl^- channel has been reported in human airway epithelia cells (Solc & Wine, 1991). After the cell was swollen, the channel was activated, and had single channel conductance of 50 pS at the holding voltage near 0 mV. This channel is sensitive to blockade with DIDS. Channels with a conductance above 200 pS have been described in several studies and it has been suggested that they are activated by mechanical stretch (Duszyk et al, 1993; Hardy & Valverde, 1994; Hamill & McBride, 1997).

1.2.3.5 Other Cl^- channels

The epithelial Cl^- channels in the apical membrane represent a diverse group of Cl^- channels. Besides the Cl^- channels described here, a variety of other novel Cl^- channels have been identified in different epithelial cells. At least three other types of Cl^- channels, with single-channel conductances of 4 pS, 10 pS, and 20 pS have been shown to have completely different biophysical properties to the Cl^- channels described here (Duszyk et al., 1989; Wilk-Blaszczak et al., 1992). These three types of Cl^- channels all had linear current-voltage relationships and were insensitive to membrane potential. The channel with a conductance of ~4 pS was shown to contribute significantly to the basal whole-cell Cl^- current in nasal epithelial cells (Duszyk et al., 1992). Similar to CFTR and ORCC, the

20 pS channel was also activated by PKA phosphorylation and was shown to be defective in CF airway epithelia (Duszyk et al., 1989). Similar 20 pS Cl^- channels with an ohmic current-voltage relationship have also been identified in CFPAC-1 cells (Duszyk et al., 1993). In addition to the 20 pS Cl^- channel, a voltage-dependent Cl^- channel with a large conductance of about 390 pS and complex kinetic behavior has also been reported in CFPAC-1 cells (Duszyk et al., 1993). Chloride channels with similar conductances have been demonstrated in other epithelia (Hanrahan et al., 1985). Besides their diverse biophysical properties, these epithelial Cl^- channels can be regulated in several ways, including G-protein, (Duszyk et al., 1995), pH (Cuppoletti et al., 1993), phosphorylation and Ca^{2+}–dependent mechanisms. This diversity of epithelial Cl^- channel types and their multiple mechanisms of regulation emphasizes the important physiological roles that chloride transport may play in epithelial tissues.

1.3 Matrix metalloproteinase in the lung

1.3.1 General introduction

Matrix metalloproteinases (MMPs), or matrixins, are a family of zinc-dependent proteolytic enzymes that break down extracellular matrix in normal physiological processes such as embryogenesis, tissue growth, and wound healing (Woessner, 1994). To date at least forteen members of the MMP family have been identified, and it is likely that some new members will be cloned in the near future. According to the substrate specificity, these MMPs can be divided into three subgroups: collagenases (MMP-1,

MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), and stromelysins and matrilysin (MMP-3, MMP-10, MMP-11, and MMP-7). The newly described membrane-type metalloproteinases, MT-MMPs, and metalloelastase (MMP-12) have not as yet been assigned to a particular group since they do not conveniently fall into one of these three categories (Ries & Petrides, 1995). The characteristics of these MMPs are summarized in Table 1-3.

Recent studies indicate that the cDNA predicted amino acid sequence of the MMPs reflects a high degree of homology between family members (O'Connor & FitzGerald, 1994). These enzymes have similar topology with three functional domains: a propeptide domain, a catalytic domain, and a C-terminal domain (Figure 1-4). All the MMPs have an N-terminal propeptide of 77 to 87 amino acids that determines the latency of the proenzyme form. This is due to the presence of a conserved sequence PRCG(V/N)PD in which the cysteine binds to the zinc of catalytic domain to maintain the stability of the latent proenzyme (Sanchez-Lopez et al., 1988). Activation of most MMPs involves sequential exogenous or endogenous cleavages of the propeptide, which destabilize the cysteine-Zn^{2+} interaction, modify the enzyme conformation, and permit further exogenous or autocatalytic processing to the final active form (Van Wart & Birkedal-Hansen, 1990).

The catalytic domain contains three histidines, acting as zinc ligands, in the sequence $HE_XGH_{XX}HS$ (Bode et al., 1993). A recent discovery indicated that MMPs all contain a second zinc atom important for the structure of the enzyme (Gooley et al.,

Table 1-3. Characteristics of the matrix metalloproteinases (adapted from Ries et al., 1995).

Enzyme names		MMP names	MW (KDa)	Matrix substrates
Collagenases	Interstitial collagenase	MMP-1	53	Collagens I, II, III, VII, X
	Neutrophil collagenase	MMP-8	85	Collagens I, II, III
	Collagenase-3	MMP-13	54	unknown
Gelatinases	Gelatinase A (72 KDa type IV collagenase)	MMP-2	72	Gelatins, collagens IV, V, Fibronectin, elastin
	Gelatinase B (92 KDa type IV collagenase)	MMP-9	92	Gelatins, collagens IV, V, elastin
Stromelysins	Stromelysin-1	MMP-3	57	Proteoglycans, gelatin, laminin, fibronectin, elastin, collagen III, IV, V, IX.
	Stromelysin-2	MMP-10	53	Same as MMP-3
	Stromelysin-3	MMP-11	55	Proteoglycans, fibronectin, gelatin, elastin, collagen IV
	Matrilysin	MMP-7	28	Proteoglycans, fibronectin, gelatin, elastin, collagen IV
Others	Metalloelastase	MMP-12	54	Elastin, fibronectin
	Membrane-type MMPs	MT-MMPs	~66	Activate MMP-2

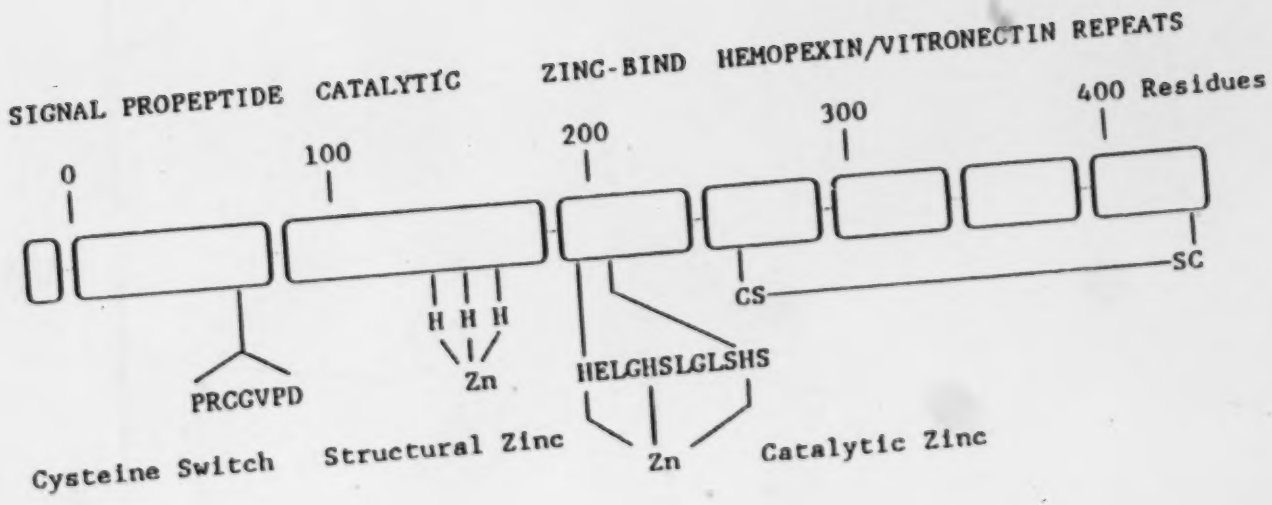

Figure 1-4. The domain structure of matrix metalloproteinase (adapted from Woessner et al., 1994).

1993). The two gelatinases contain an additional 175-residue insert in the catalytic region, which is believed to assist in binding of gelatinases to their substrates (Woessner, 1994). The exact mechanism of the interaction of MMPs and their substrates is not yet clear. Between the zinc-binding domain and the hemopexin-like domain is a variable hinge region, rich in proline that may permit the C-terminal domain to fold back on the catalytic domain. Matrilysin, the smallest of the MMPs, is composed of a propeptide and catalytic domains only (O'Connor & FitzGerald, 1994).

The C-terminal domain consists of four repeats that have weak homology to hemopexin and fibronectin. The amino end of this domain contains a hinge region and a disulfide bridge connects its extreme ends. This domain plays a part in determining the substrate specificity of the collagenases and stromelysins, and is also involved in the interaction of MMPs with the tissue inhibitors of metalloproteinase, TIMP-1 and TIMP-2 (Murphy et al., 1992a, 1992b). Various chimeras have been produced by manipulation of the cDNA whereby the C-terminal domains of various matrixins are interchanged. The C-domain of interstitial collagenase can cause the catalytic domain of stromelysin-1 to bind to collagen, but does not confer the ability to digest collagen (Murphy et al., 1992a). Collagenase with the stromelysin C-domain also fails to digest collagen. However, collagenase activity depends not only on the C-domain but also on specific sequences within the zinc-binding domain (Sanchez-Lopez et al., 1993). The C-domain was also reported to modulate the binding of the active centers of the MMPs to tissue inhibitors of MMPs (TIMPs), the major local inhibitors of MMPs (Murphy et al., 1992a). There are two distinct domains in TIMP, of which the C-domain binds the C-domain of gelatinase

so that the N-domain is free to interact with other matrixins or with the active center of the same molecule when it becomes active (Willenbrock et al., 1993).

MMPs are detected in a wide range of tissue cells such as stromal cells, connective tissue cells, and white blood cells. Recent reports showed an expression of MMPs in epithelial cells including airway submucosal gland serous cells (Tournier et al., 1994). The enzymes are responsible for the proteolytic degradation of extracellular matrix components such as basement membrane or interstitial stroma. Therefore, they play a crucial role in normal physiological processes including morphogenesis, cartilage and bone repair, wound healing, angiogenesis, physiological cell migration and tissue remodeling. Most recently, it has been found that MMPs are also involved in processes not directly related to tissue remodeling, such as platelet aggregation (Sawicki et al., 1997) and degranulation of airway gland cells (Sommerhoff et al., 1996). The malfunction of MMPs can also result in the exacerbation of inflammation, and cause diseases such as myocardial infarction, liver fibrosis, and emphysema.

MMPs are tightly regulated at different levels due to their high biological activities as discussed above. At the transcriptional level, MMP expression is precisely controlled by various cytokines acting through positive or negative regulatory elements of its genes (Ries & Petrides 1995; Wu et al., 1997) (Table 1-4). Moreover, pharmacological modulation of MMP production is observed with retinoids and corticosteroids (Clark et al., 1987; Brinckerhoff et al., 1991). Both decrease transcription of interstitial collagenase and stromelysin-1. The retinoids also enhance TIMP-1 production (Clark et al., 1987). At the protein level, MMP activity is regulated by activation of the latent enzymes and

Table 1-4. Effects of Cytokines and Other Mediators on the Production of MMPs and TIMPs (adapted from Ries et al., 1995).

Mediator	Collagenase	Stromelysin	Gelatinases		Inhibitors	
	MMP-1	MMP-3	MMP-2	MMP-9	TIMP-1	TIMP-2
TPA	⇑	⇑	⇔	⇑	⇑	⇔
TNF-α	⇑	⇑	⇔	⇑	⇑	⇔
Il-1	⇑	⇑	⇔	⇑	⇑	⇔
EGF	⇑	⇑		⇑		
PDGF	⇑	⇑				
TNF-β	⇑		⇔	⇑		
bFGF	⇑			⇔		
TGF-β	⇓	⇓	⇑	⇑	⇑	
Il-4	⇓			⇓	⇔	
INF-γ	⇓	⇓		⇓		
Retinoic acid	⇓	⇓			⇑	
Dexamethasone	⇓	⇓				

Arrows indicate a stimulatory effect (⇑), an inhibitory effect (⇓) or no effect (⇔) of the respective regulator.

interaction with endogenous and exogenous inhibitors. Enzymes including plasmin, trypsin, kallikrein, neutrophil elastase and cathepsin G activate MMPs by cleaving the propeptide of MMP (Murphy et al., 1994; Mignatti & Rifkin, 1996). Reagents such as organomercurials and detergents activate MMPs by disrupting the cysteine-Zn^{2+} bond. More recently, the membrane type matrix metalloproteinases are reported to be an important activator of MMPs (Sato et al., 1996; Okada et al., 1997; Butler et al., 1997). On the other hand, MMPs can be inhibited by endogenous inhibitors including specific tissue inhibitors of metalloproteinases (TIMPs) and non specific inhibitors such as α_2-macroglobulin in vivo. In vitro, MMPs can be inhibited by specific drugs such as 1,10-phenanthroline.

1.3.2 MMPs in lung physiological and pathological processes

MMPs are produced by virtually all resident lung cells including fibroblasts, alveolar macrophages, epithelial and endothelial cells (O'Connor & FitzGerald, 1994). In the healthy lung MMPs are involved in normal extracellular matrix turnover and also participate in wound healing. The activity of these enzymes is tightly controlled to maintain the balance between synthesis and degradation of the extracellular matrix component. This dynamic balance is of extreme importance for the normal lung development and the maintenance of the extracellular matrix scaffold, which supports the alveolar structure and is essential for normal lung function (Tetley, 1993). Given their role in normal lung homeostasis, it is not surprising that the MMPs are implicated in a range of pulmonary diseases characterized by alterations in alveolar structure, or abnormal wound healing responses. These diseases include emphysema, adult respiratory

distress syndrome, interstitial fibrosis, granulomatous disease, lung cancer and pleural disease (Petty, 1991; D'Armiento et al., 1992; O'Connor & FitzGerald, 1994). Recent studies also indicate an imbalance between 92 KDa gelatinase and TIMPs in the sputum of patients with cystic fibrosis (Delacourt et al., 1995), and a reduced activity of MMPs in asthmatic subjects (De Gouw et al., 1996).

All the roles MMPs play in the lung discussed here are directly related to their common actions via extracellular matrix degradation. Recently, it was demonstrated that MMPs also play a regulatory role in the degranulation of airway submucosal gland cells (Sommerhoff et al., 1996). The finding that metalloprotease inhibitors block degranulation induced by histamine and bradykinin indicates that a metalloprotease is involved in the stimulation-secretion coupling mechanisms in serous cells of airway submucosal gland. The inhibitors also block degranulation elicited by cAMP analogues and the Ca^{2+} ionophore, suggesting that this enzyme is required in a late step of the signal transduction cascade, i.e., after Ca^{2+} entry. The major metalloprotease detected by gelatin-zymography in serous cell-conditioned medium was identified as a proenzyme of gelatinase A (72-KDa gelatinase or MMP-2). The proenzyme was activated by the cleavage of chymase, tryptase, or progelatinase A per se. However, the mechanism underlying the regulatory process of degranulation by gelatinase A is not yet clear. This marked regulatory action may suggest a role for metalloproteinases in the pathogenesis of excessive airway secretions commonly associated with inflammatory airway diseases such as chronic bronchitis and cystic fibrosis.

Chapter 2 Outline of the thesis

2.1 Specific objectives

The main objective of this thesis was to study the role of MMPs in the regulation of Cl^- ion movements in human airway epithelial cells. My goal was to investigate the possibility of the regulation of MMP activity affecting transepithelial Cl^- ion movement and alter the viscoelastic properties of the mucus. My specific aims were 1) to identify the MMPs produced and secreted by human airway epithelial cells, and 2) to investigate if regulation of MMP activity has an effect on Cl^- ion movement.

2.2 Rationale

As discussed in Chapter I, MMPs play a crucial role in physiological and pathological processes by stimulating the remodeling of the extracellular matrix. The main source of MMPs in the lung are stromal cells including fibroblasts, endothelial cells, osteoblasts, keratinocytes and chrondrocytes. Recently it has been shown that tracheal gland epithelial cells secrete MMP-2 (gelatinase A, 72 KDa type IV collagenase) which plays a crucial role in extracellular matrix remodeling and tracheobronchial gland morphogenesis (Tournier et al., 1994). While these functions of MMPs are well established, there is some evidence to suggest that MMPs may also play a role in processes not related to tissue remodeling. First, MMPs were shown to affect the viscoelasticity of mucus and thus contribute to the pathology of cystic fibrosis

(CF)(Delacourt et al., 1995). Secondly, MMP-2 was shown to affect degranulation of human airway gland cells (Sommerhoff et al., 1996). Since submucosal glands are the major expression sites of cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels that contribute to mucus secretion, it is possible that MMPs could be involved in the regulation of these channels, and thus affect submucosal gland mucus secretion. Therefore, in order to address this question I explored the secretion of MMPs in airway epithelial cells and studied their role in regulation of chloride channels.

2.3 Experimental design

In this study, two human airway epithelial cell lines, A549 and Calu-3 were used. Calu-3 cells, derived from submucosal glands, have been shown to be a useful model in the study of Cl^- secretion and other functions of airway epithelial cells (Shen et al., 1994). They show cAMP-dependent Cl^- secretion mediated mainly by CFTR Cl^- channels (Haws et al., 1994; Singh et al., 1997). This is in agreement with the observations that serous cells of the submucosal glands express high levels of CFTR (Engelhqrdt et al., 1992) and they play a key role in mucosal defenses, by controlling the properties and amount of gland secretions (Nadel, 1991). For comparison, A549 cells were used since they do not express endogenous CFTR and thus are particularly suitable for studying non-CFTR Cl^- conductance (Renier et al., 1995). In contrast to the cells from primary culture, the two human airway epithelial cell lines Calu-3 and A549 cells do not undergo crisis and retain constant properties over repeated passages. Therefore, they are a continuous supply of the epithelial cells for this study.

To investigate the role of MMPs in the regulation of airway epithelial Cl^- channels, firstly we evaluated the presence of the two gelatinases, MMP-2 (gelatinase A or 72 KDa gelatinase) and MMP-9 (gelatinase B or 92 KDa gelatinase) in the two human airway epithelial cell lines, Calu-3 and A549 cells. There are several reasons for choosing MMP-2 and MMP-9 out of all known fourteen MMP members. First, MMP-2 and MMP-9 are the most probable MMPs to be expressed by airway surface epithelium and submucosal gland serous cells, because airway epithelial cells overlie the subepithelial basal lamina, which is predominantly composed of types IV and V collagen and laminin, while these matrix are the major substrates of gelatinases (Yao et al., 1996). Secondly, the expression of MMPs in the reported studies of airway epithelial cells and submucosal gland serous cells so far, are suggested to be related to these two gelatinases. Thirdly, gelatin zymography can be used in analyzing the expression and secretion of gelatinases and their enzyme activities can also be determined by this technique. Finally, we have polyclonal antibodies against human MMP-2 and MMP-9 at hand, which are very useful for immunoblotting study to detect these enzymes in the two cell line cells.

Western blot analysis was performed to determine if Calu-3 and A549 cells constitutively produce the two gelatinases, MMP-2 and MMP-9, and tissue inhibitor of MMP-2 (TIMP-2). The rabbit polyclonal antibodies against human MMP-2 or MMP-9, and monoclonal antibodies against TIMP-2 were used to recognize the two gelatinases and TIMP-2 respectively, to determine if Calu-3 and A549 cells produced them. The antibodies in the immunoblotting recognized both proenzyme and active forms of the gelatinases. Gelatin zymography was employed to determine if these two human airway epithelial cell line cells secrete gelatinolytic enzymes MMP-2 and MMP-9. The

gelatinolytic activities of these enzymes were also quantified by this enzymatic method. Both proenzyme and active forms could be detected by the gelatin zymography method.

The perforated whole-cell voltage clamp technique has been proved to be a very useful method in the study of ion channel properties and regulation. This technique is valuable in the study of the modulation pathway of ion channels since it bears the following advantages: 1), the current under examination can be monitored continuously; 2), drugs can be applied directly into the bath solution or through the patch pipette; 3), the perforated patch can prevent the dialysis of cytoplasm that occurs with conventional whole-cell patch recording.

In this study, the amphotericin B-perforated whole-cell voltage clamp technique was used to investigate the effects of MMP-2 and MMP-9 on the whole-cell Cl^- currents in Calu-3 cells and A549 cells. The MMP inhibitor, 1,10-phenanthroline was applied into the bath solution and the currents before and 5 minutes after the addition of 1,10-phenanthroline were compared and analyzed. Since 1,10-phenanthroline is nonspecific to the MMP members, antibodies against MMP-2 and MMP-9 were applied to determine the effects of these two gelatinases on the whole-cell Cl^- currents. This experiment also helped to decide if the action of MMPs locates to the intracellular or extracellular side since 1,10-phenanthroline is a membrane permeable chemical while antibodies do not cross the cell membrane. Human recombinant MMP-2 (rMMP-2) was applied to the extracellular side to provide direct evidence that MMP-2 participates in the regulation of airway epithelial Cl^- channels. To further confirm that the Cl^- current affected by MMPs was mainly conducted by CFTR Cl^- channels, specific CFTR inhibitor, diphenylamine-2-carboxylate (DPC), was applied after the addition of 1,10-phenanthroline.

On the basis of the results of whole-cell patch clamp study, single channel patch clamp procedures with inside-out configuration were performed to further identify the channels affected by MMPs. This method provides more specific information, such as single channel conductance, and allows the study of single channel characteristics, thus leading to the identification of the Cl^- channels regulated by MMPs. Besides, this method can also help to investigate the possible mechanism underlying the interaction of MMPs and CFTR.

Chapter 3 Materials and Methods

3.1 Materials

Dulbecco's modified Eagle's medium (DMEM), Eagle's minimum essential medium (EME), porcine skin gelatin, p-aminophenylmercuric acetate (APMA), Coomassie brilliant blue G-250, 1,10-phenanthroline, amphotericin B, and diphenylamine-2-carboxylate (DPC) were obtained from Sigma. Fetal bovine serum was purchased from HyClone Lab. (Logan, Utah). Monoclonal antibodies against TIMP-2 (clone Ab-1) were obtained from Oncogene Science (San Diego, CA). Polyclonal antibodies against human MMP-2 or MMP-9 were a generous gift from Dr. G. Sawicki (U of A). The IgG from rabbit serum was purified using Affi-Prep Protein A Support (Bio-Rad) affinity chromatography, and the resultant preparation was stored at –20 °C. Human recombinant MMP-2 was a kind gift from Dr. M. Radomski (U of A) and was activated by 2 mM APMA in 50 mM Tris-HCl buffer (pH 7.5) with 0.15 M NaCl and 5 mM $CaCl_2$ for 2 hours at 22 °C (Tournier et al., 1994).

3.2 Cell preparation

Calu-3 and A549 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD), and were maintained in culture medium, the 1:1 mixture of DMEM and EME. Each medium was buffered with $NaHCO_3$ at a concentration of 0.37% W/V for DMEM and 0.22% W/V for EME and supplemented with 10% fetal bovine

serum (FBS). The final pH of the medium was adjusted with hydrochloric acid (HCl) and sodium hydroxide (NaOH) to 7.4. The culture was kept at 37 °C in a humidified 5% CO_2-95% air incubator and medium was replaced with fresh medium every two days. Confluent monolayers were subcultured by treatment with 0.05% W/V trypsin-0.02% ethylene-diaminetetra-acetic acid (EDTA) W/V in Ca^{2+}- and Mg^{2+}-free Hank's buffered salt solution at 37 °C for 15-20 minutes. Cells were then washed with the growth medium to remove trypsin residues and were recollected by centrifuging the cell suspension at 200 g for five minutes. For maintaining the cell line, cells were either kept growing by reseeding them in the growth medium at a plating density of 1 x 10^5 cells / ml or put into the culture medium with 5% (V/V) dimethyl sulfoxide (DMSO) and frozen in the liquid nitrogen. Cells were prepared then for electrophysiological recordings, zymography and Western blot studies. The cells used for these studies were from passages twenty to thirty for Calu-3 cells and passages seventy-two to eighty-five for A549 cells.

For electrophysiological recording, cells were routinely trypsinized and plated at a density of 1 x 10^4 cells / ml in 35-mm petri dishes (Falcon, Franklin Lakes, NJ) and were used within twenty-four hours after seeding. Before the patch clamp recording, cells were gently rinsed with bath solution three times.

For western blot and zymography studies, cells were trypsinized and plated at a density of 1x 10^6 cells / ml in a 25 T flask (Falcon, Franklin Lakes, NJ) and grown to greater than 90% confluence. The old medium was decanted and the cells were washed with the FBS-free medium three times. Then the cells were incubated in 3ml FBS-free culture medium at 37 °C in standard cell culture conditions described above. After 48 hours incubation, the supernatant (Calu-3 cell- or A549 cell-conditioned medium) was

collected and centrifuged at 800 g for 10 minutes to get rid of the cell residues. The Serum-free medium conditioned by A549 or Calu-3 cells (1 x 10^6 cells/ml) was then concentrated 30 times by spinning the medium at 3020 g for about 3 hours at 4 °C using Centricon-10 membranes (Amicon). The cells were then scratched down from the bottom of flasks and collected for Western blot analysis. All the samples, Calu-3 cells and cell-conditioned medium, and A549 cells and cell- conditioned medium were stored at –80 °C until use.

A human fibrosarcoma cell line, HT-1080, was obtained from ATCC and grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS and antibiotics. These cells were used as a reference for zymography and Western blot studies, since they are known to secrete large amounts of MMPs (Emmert-Buck et al., 1995).

3.3 Gelatin-zymography

The Calu-3 cell- and A549 cell-conditioned serum-free medium were concentrated thirty times and resolved by 7% SDS-PAGE in the presence of 2 mg/ml porcine skin gelatin. The methods of Laemmeli (Laemmeli, 1970) were followed, excluding any reducing agents or boiling procedures. After electrophoresis at 4 °C, the gel was washed for 1 hour (3 times, 20 minutes each) in 2.5% Triton X-100 at room temperature to remove SDS. The gel was then incubated at 37 °C in reaction buffer (50 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.05 NaN_3, pH 7.6) for 48 hours. After incubation, the gel was stained for about 1 hour with 0.05% Coomassie Brilliant Blue G-250 in a mixture of methanol: acetic acid: water (2.5: 1: 6.5) and subsequently de-stained in 4% ethanol

and 8% acetic acid mixed solution. Gelatinolytic activities were detected as transparent bands against the blue background of Coomassie brilliant Blue-stained gelatin. The dependence of gelatinase activity on the presence of divalent cations such as Ca^{2+} and Zn^{2+}, was determined by incubating the gels overnight with 20 μM 1,10-phenanthroline in the buffer. The activities of the detected enzymes were determined by scanning the gels and quantifying the intensities of the separate bands using SigmaGel software (Jandel Corporation, San Rafael, CA). Results were expressed as arbitrary units.

3.4 Western blot analysis

Samples of cell homogenates (20 μg protein) were subjected to 7% SDS-PAGE. Following electrophoresis, samples were electroblotted onto polyvinylidene fluoride membranes (Schleicher and Schuell, Keene, NH) and probed with antibodies against MMP-2 (1 μg/ml), MMP-9 (1 μg/ml), or TIMP-2 (5 μg/ml). Bands corresponding to MMP-2, MMP-9 or TIMP-2 were detected with an ECL kit (Amersham).

3.5 Patch Clamp Studies

The techniques of perforated whole-cell voltage clamp and single channel patch clamp with inside-out configuration were employed in this study basically according to the description by Hamill et al. (1981). The schematic representation of this technique is shown in Figure 3-1.

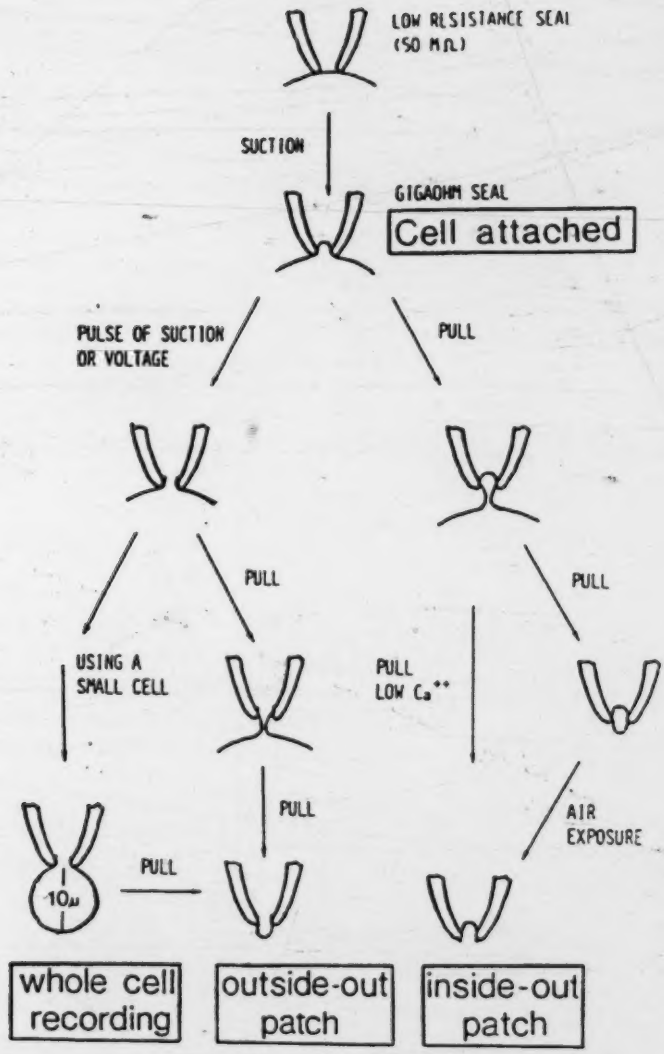

Figure 3-1. Schematic representation of the patch clamp technique. The patch clamp technique consists of two versions: the whole cell and the single channel version. The single channel version can be further divided into cell attached, outside-out patch and inside-out patches (adapted from Hamill et al., 1981).

3.5.1 Perforated whole-cell voltage clamp technique

The amphotericin B-perforated whole-cell voltage clamp was performed to measure the whole cell Cl^- current according to the method introduced by Rae et al (1991). Experiments were performed on single cells using pipettes made from thin-walled borosilicate microfilament glass (A-M Systems Inc, Everett, WA). The pipettes were made using a Sutter Instruments (Novato, CA) Model P-87 electrode puller and their tips were fire-polished to the final resistance of 3-5 MΩ immediately before experiments. Cells were washed three times with the bath solution and bathed with 2-3 ml bath solution before experiments. The bath solution contained (in mM): 140 NaCl, 5 KCl, 0.5 $MgCl_2$, 1 $CaCl_2$, and 10 4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid (HEPES) (pH 7.4). The pipette was filled with pipette solution in two steps: firstly the tip was briefly dipped in the pipette solution and then back filled the pipette with pipette solution containing 240 μg/ml amphotericin B (Sigma Chemical Company, St. Louis, MO). The pipette solution contained (in mM): 5 NaCl, 140 KCl, 1 $MgCl_2$, 0.2 $CaCl_2$, and 10 HEPES (pH 7.4) and 0.5 ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N', -tetraacetic acid (EGTA). Both bath solution and pipette solution was filtered through filters with pore size of 0.22 μm in diameter before use. The pH of the solutions was titrated to 7.4 using HCl and NaOH. Whole-cell Cl^- currents were recorded with a List EPC-7 patch-clamp amplifier (Adams & List, Germany). Before the cell membrane was touched, the junction potential between pipette and bath solution was zeroed by adjusting the pipette current to zero. After a giga-ohm seal was formed, 15-20 minutes were allowed to ellapse until the current became stable. Before recording, C-slow and G-series

controls were adjusted to cancel the transient capacitance current. During whole-cell voltage clamp, the voltage was controlled and an IBM compatible computer, using 12-bit digital-to-analog and analog-to-digital converters, collected the current data. The holding potential was –40 mV. Then, different voltages from -70 to 70 mV in 20-mV step were applied for 200 ms. Steady state currents in last 15 ms are measured from each step to generate the current-voltage (I-V) relation. The series resistance of patch and cell capacitance was measured directly by the compensation circuitry of the patch clamp amplifier. In some experiments, cell membrane capacitance C_m, was estimated in the absence of hardware compensation, from the integral of the transient current response I, to V=10 mV voltage step V: $C_m = 1/V \int_0^t I(t)dt$. Series resistance R_s, was then calculated by fitting the relaxation of the capacitive transient to a single exponential to find the time constant τ, and then calculating R_s from the relationship $R_s = \tau/C_m$. All experiments were performed at room temperature (21± 2 °C).

3.5.2 Single channel patch clamp technique

Standard single channel patch clamp studies with inside-out configuration were carried out according to the procedure described by Hamill et al. (1981). The cell preparation was similar to the whole-cell voltage clamp measurement. The pipettes were made the same way as those for whole-cell recording but the tips were fire-polished to larger open resistance between 10-15 MΩ and coated with Sylgard (Dow Corning) to reduce noise. Standard pipette solution contained (in mM) 140 choline Cl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES (pH 7.4). Standard bath solution contained 140 NaCl, 1 $MgCl_2$, 1

$CaCl_2$, 10 HEPES (pH 7.4). After a gigaseal was formed, the sealed pipette was carefully lifted to expose to air and quickly returned to the bath solution. This step excised an isolated membrane patch with inside-out configuration. Single channel current was detected with List EPC-7 amplifier. The pipette electrode was set to command voltage and the bath electrode was held at virtual ground. The EPC-7 output signal was filtered at 3 KHz (8-pole Bessel filter, Frequency Devices Inc., Haverhill, MA, USA), monitored on a storage oscilloscope and stored in digital format on a videotape (Bio logic Science Instrument). For all single channel records analyzed, the applied membrane potential was given as the pipette relative to the grounded bath electrode. All experiments were performed at room temperature (21 ± 2 °C).

The procedures used for data analyses were based on those described by Colquhoun and Sigworth (1985). The half-amplitude criterion was used as a threshold to distinguish between open and closed states. Event durations were corrected for filter rise-time by a polynomial approximation (Colquhoun & Sigworth, 1985). Only openings longer than the filter dead time were used to compute the mean channel current amplitude.

3.6 Statistical Analysis

The data is expressed as mean ± standard error of the mean (SEM) unless where is specifically mentioned. The steady state Cl^- currents at clamping voltages +70 mV and – 70 mV, obtained from current voltage relationships were used to determine the effect of the test agents. Student's *t*-test was used for statistical analysis between two group means.

Statistical differences among multiple group means were determined using one-way analysis of variance (ANOVA), and $P<0.05$ was considered statistically significant.

Chapter 4 Results

4.1 The expression of MMPs by Calu-3 and A549 cells

4.1.1 The secretion of MMP-2 and MMP-9 by Calu-3 and A549 cells

Zymography was used to determine whether A549 and Calu-3 cells secreted gelatin-degrading enzymes. A human fibrosarcoma cell line, HT-1080, was used as standard, since they are well known to secret large ammount of both MMP-2 and MMP-9. Figure 4-1A shows that gelatinolytic activities investigated in both Calu-3 cell- and A549 cell-conditioned medium were detected in two forms: a major band at 72 KDa corresponding to the proenzyme form of MMP-2; and a minor band at 92 KDa corresponding to the proenzyme form of MMP-9. Two active forms of MMP-2 and MMP-9, respectively, were expected to be detected in the zymograms for both cell lines. However, only one band corresponding to the active form of MMP-2 at 68 KDa was found in A549 cell-conditioned medium, and none found in Calu-3-conditioned medium. The gelatinolytic activity of MMP-2 and MMP-9 was completely inhibited in the presence of 20 μM 1,10-phenanthroline (Figure 4-1B). Identical gelatinase patterns were obtained with repetitive samples (n=6).

Quantification of these gelatinolytic bands through image analysis revealed that both Calu-3 and A549 cells released large amounts of MMP-2 and small amounts of MMP-9. The activity of MMP-2 secreted by A549 cells was about three fold that secreted by Calu-3 cells (Figure 4-2).

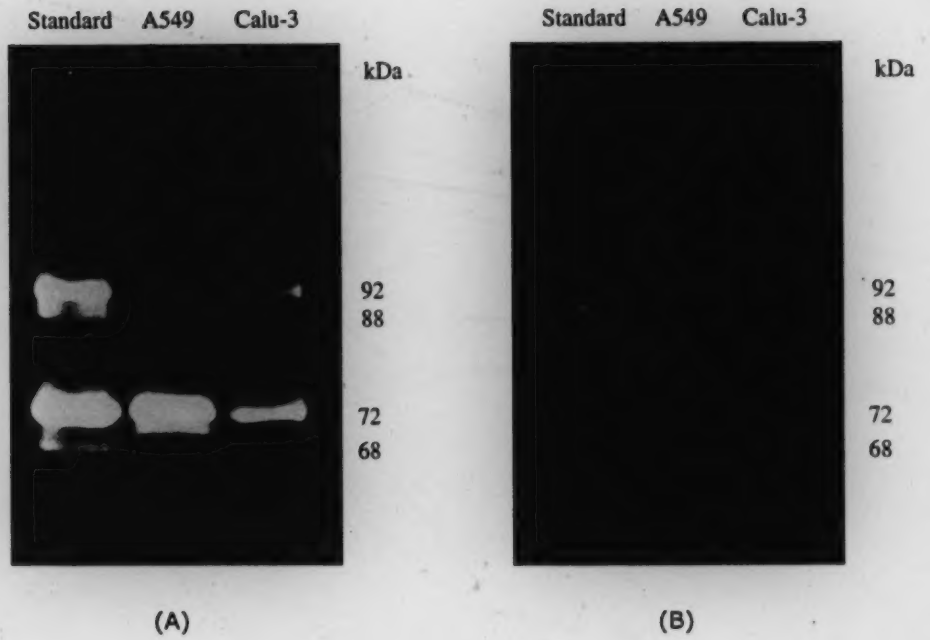

Figure 4-1. The secretion of gelatinases by A549 and Calu-3 cells and identification as metalloproteinases. The gelatin-degrading activities in A549- and Calu-3-conditioned medium were detected by gelatin-zymography (A). The gelatinolytic activity was inhibited by incubating the zymograms in the presence of 20 μM phenanthroline (B).

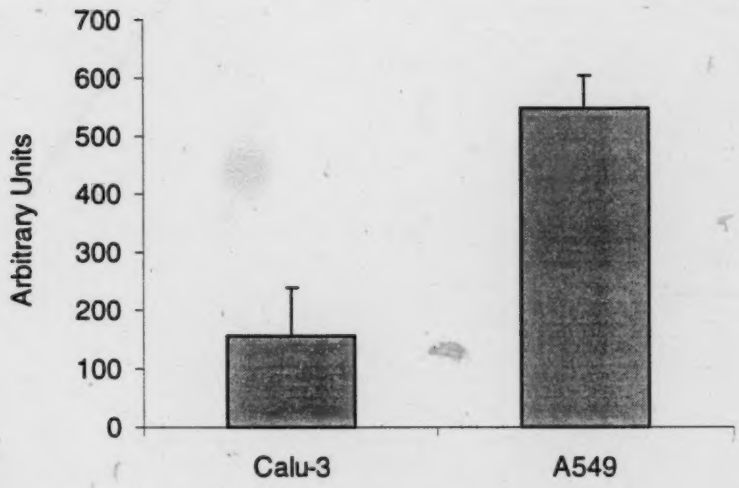

Figure 4-2. The relative value (mean ± S.D., n=6) of the proenzyme form of MMP-2 secreted by A549 and Calu-3 cells. The cells were conditioned in FBS-free medium for 48 hours and analyzed by gelatin-zymography.

The expression of MMP-9 was on the boarder of detection. In some experiments, no MMP-9 was seen under standard zymography condition, whereas after longer exposure (e.g. prolonged incubation time for gelatin degrading from 24 to 48 hours), a faint band corresponding to MMP-9 could be identified. However due to its low expression level, it would be difficult to perform correct statistical analysis of expression and no such calculation was attempted.

4.1.2 The presence of MMP-2 and MMP-9 in Calu-3 and A549 cells

In order to determine more specifically whether Calu-3 and A549 cells express MMP-2 and MMP-9, polyclonal anti-MMP-2 and anti-MMP-9 antibodies were used to recognize these two gelatinases in the cell lysate and the result is shown in Figure 4-3. The standard, HT1080, was the same as used in the zymography study. A major band at 72 KDa was detected in both Calu-3 and A549 cell lysate indicating that large amounts of latent MMP-2 was expressed in both Calu-3 and A549 cells. A minor band at 68 KDa corresponding to the active form of MMP-2 was detected only in A549 cell lysate. A minor band at 92 KDa was recognized by anti-MMP-9 antibodies, indicating both A549 cells and Calu-3 cells express small amounts of MMP-9 but no active forms were detected in the cell lysate. Identical blots were obtained with repetitive samples (n=7).

The monoclonal antibodies against tissue inhibitor of matrix metalloprotease-2 (TIMP-2) were also employed to determine whether these two cell lines produce TIMP-2. The results are shown in Figure 4-3. A minor band at 21 KDa corresponding to TIMP-2 was detected only in Calu-3 cell lysate (Figure 4-3).

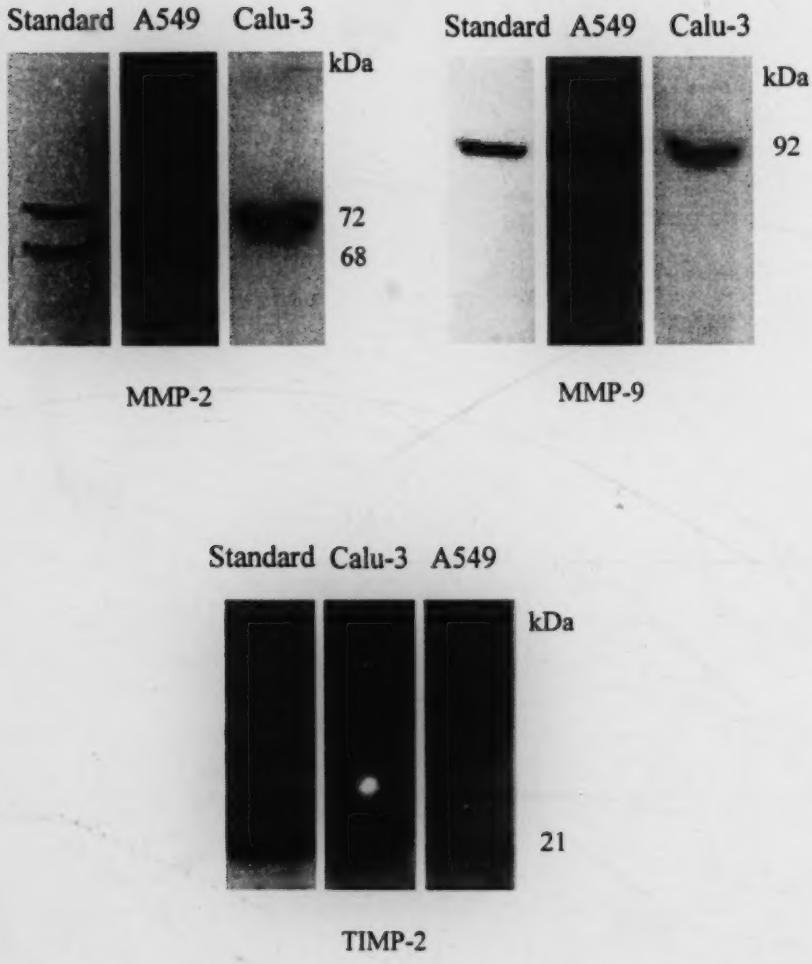

Figure 4-3. Presence of MMP-2, MMP-9, and TIMP-2 in A549 and Calu-3 cells. Western blot analysis of MMP-2 (top left), MMP-9 (top right), and TIMP-2 (bottom) produced by A549 and Calu-3 cells.

4.2 The effect of MMPs on whole-cell Cl^- current

Perforated patch clamp method with the whole-cell configuration was used to study the effects of the MMP inhibitor 1,10-phenanthroline, antibodies against MMP-2 or MMP-9, and human recombinant MMP-2 on the whole-cell Cl^- current. This modification of the whole-cell technique requires the presence of a pore-forming antibiotic in the pipette solution before formation of a cell-attached patch. In this studies amphotericin B, which forms pores in the cell membranes with an effective diameter of about 0.8 nm, was used. The pore is formed by two-half pores, each consisting of eight antibiotic and eight sterol molecules (Franciolini & Petris, 1992). Normally, this antibiotic forms anion selective pores with a conductance of about 6.5 pS in 2 M NaCl. However, monovalent cations can also permeate the channel, but only in the presence of a permeant anion. During the experiments pores formed by amphotericin B were permeable for both anions and cations. Under these conditions perforation of the cell membranes creates an electrical continuum (i.e. equilibrium of monovalent ions such as Cl^- across the membrane) for recording of Cl^- currents without significant disruptions of cellular structural elements or dilution of second messengers or ATP within the cytosol, which usually occurs in conventional recording (Rae et al., 1991). In this study, the average capacitance was 22.56 ± 5.51 pF (n=28) for Calu-3 cells and 16.49 ± 5.80 pF (n=11) for A549 cells. The average series resistance was 9.8 ± 5.2 MΩ (n=16). To accommodate for the variations in cell size, currents were normalized as a current density and expressed as pA/pF. All experiments were performed at room temperature (21± 2 °C).

4.2.1 The effect of 1,10-phenanthroline on whole-cell Cl^- currents

4.2.1.1 The effect of 1,10-phenanthroline on whole-cell Cl^- currents in Calu-3 cells

To investigate the effects of 1,10-phenanthroline on the whole-cell Cl^- current, 1,10-phenanthroline was applied to the bath solution. There was a significant increase of whole-cell Cl^- current of Calu-3 cells 5 minutes after the addition of 1,10-phenanthroline at concentrations of 5, 10, and 100 μM in a dose-dependent manner. The maximum increase occurred at concentrations greater than 10 μM while no effect was observed at 1 μM ($n=6$, $p>0.05$). The steady state Cl^- current at a clamping voltage of +70 mV was enhanced by 58.33 ± 7.73 % ($n=6$, $p<0.05$) at 100 μM, and 57.85 ± 8.01 % ($n=7$, $p<0.05$) at 10 μM. At 5 μM, Cl^- current was increased by 27.25 ± 4.58 % ($n=9$, $p<0.05$). Similar results were observed at a clamping voltage of –70 mV. The typical whole-cell recordings before and after the addition of 5 μM 1,10-phenanthroline and their corresponding current-voltage curves (I-V curves) are shown in Figure 4-4. Figure 4-4D also shows a linear I-V relationship in a time and voltage independent manner in both basal condition and that after the stimulation of 1,10-phenanthroline, indicating that the majority of Cl^- current in Calu-3 cells is conducted by CFTR. This is consistent with the previous observation that CFTR Cl^- channels dominate whole-cell current in Calu-3 cells. Under basal condition, the Calu-3 cells have a current density of 16.96 ± 3.43 pA/pF ($n=28$) at clamping voltage +70 mV and 16.18 ± 3.97 pA/pF ($n=28$) at –70 mV. The current density corresponding to these recordings was plotted in Figure 4-5.

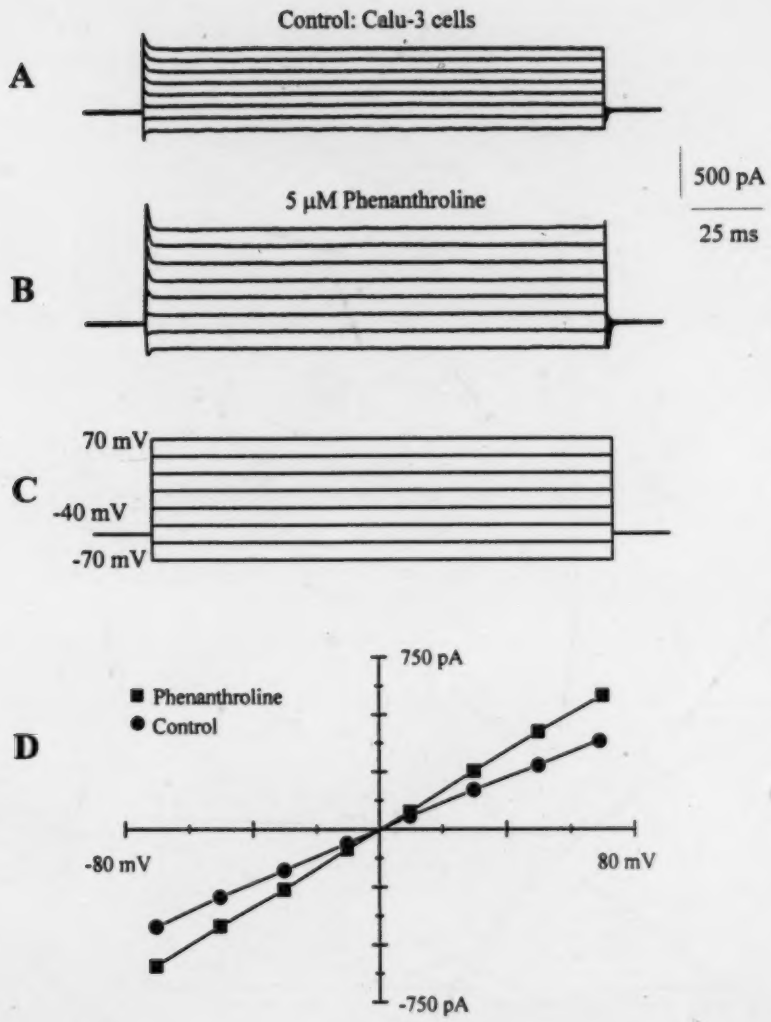

Figure 4-4. Typical recordings of whole-cell Cl^- current in Calu-3 cells in the absence (A) and presence (B) of 5 μM 1,10-phenanthroline. The transient currents in each voltage step at the beginning of recordings A and B are capacitance currents due to the incomplete compensation. C). Applied voltage protocol. The same voltage protocol was used in all other whole-cell patch clamp studies. D). Current-voltage relationship corresponding to recordings shown in panels (A) and (B).

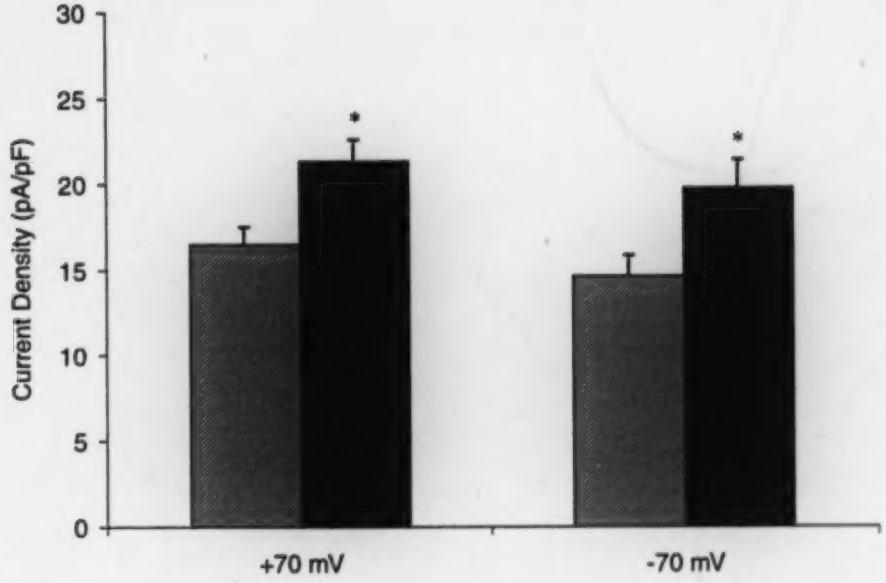

Figure 4-5. The effect of 1,10-phenanthroline on whole-cell Cl^- current in Calu-3 cells. Shadowed bars: control; gray bars: current density after the treatment of 5 μM 1,10-phenanthroline (n=9). Asterisks (*) indicate statistically significant differences between control and experiments ($p<0.05$).

Plot of the relative current (relative to the control) versus phenanthroline concentrations for clamping voltages of +70 mV (Figure 4-6) and –70 mV (Figure 4-7) clearly showed the dose-dependent stimulatory effects of 1,10-phenanthroline on whole-cell Cl^- current in Calu-3 cells.

4.2.1.2 The effect of 1,10-phenanthroline on whole-cell Cl^- currents in A549 cells

For comparison, 1,10-phenanthroline was applied to the bath solutions of A549 cells to determine whether it would affect the whole cell Cl^- currents of A549 cells. The results showed that the treatment of 1,10-phenanthroline at the concentration of either 5 or 10 μM did not affect the Cl^- channel activities of A549 cells. The results of 1,10-phenanthroline on whole-cell Cl^- current in A549 cells are shown by plotting the relative current (relative to the control) at varying concentrations of phenanthroline. These results, along with the statistic significance, are summarized in Table 4-1. Figure 4-8 shows that typical Cl^- current trace in A549 cells recorded before and 5 minutes after the application of 5 μM 1,10-phenanthroline along with their I-V relationship. From Figure 4-8C, the I-V curve shows clearly outwardly rectifying properties for both basal and experimental conditions, indicating that A549 cells are dominated by non-CFTR Cl^- channels (e.g. ORCC, stretch-activated Cl^- channels). The cells in this study have a basal Cl^- current density of 16.11 ± 1.67 pA/pF (n=11) at the clamping voltage of +70 mV and 7.52 ± 1.45 pA/pF (n=11) at the clamping voltage of –70 mV. Figure 4-9 showed the normalized current density at clamping voltage of +70 mV and –70 mV before and after the addition of 5 μM 1,10-phenanthroline.

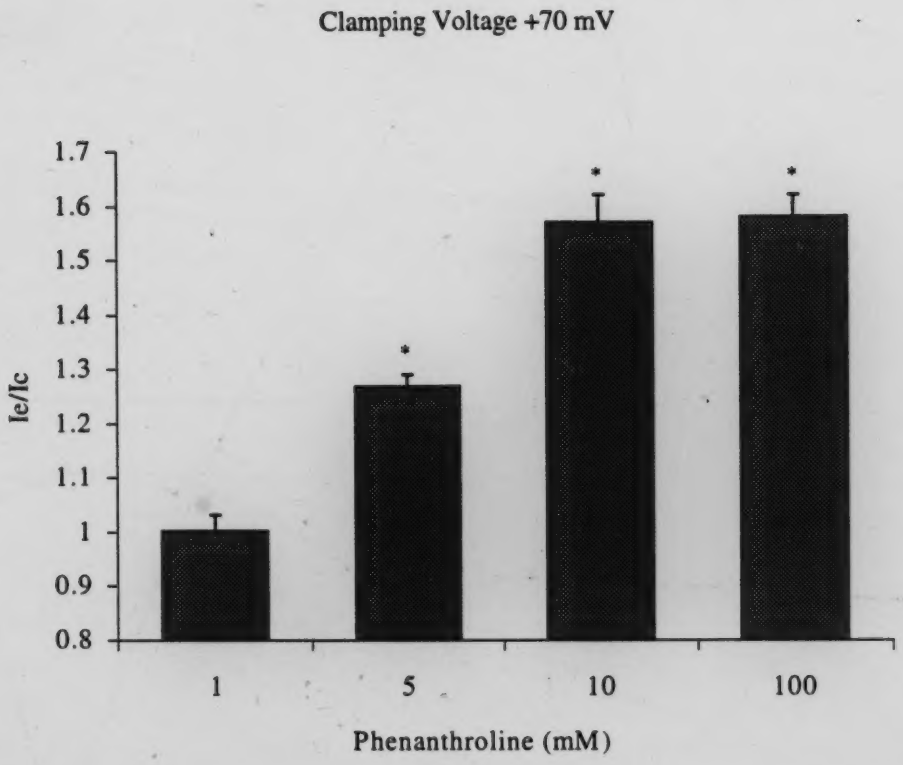

Figure 4-6. Dose-dependent stimulatory effects of 1,10-phenanthroline on whole-cell Cl^- current in Calu-3 cells. The histograms show the relative current to control at clamping voltage of +70 mV as phenanthroline concentration is increased (n= 6 for phenanthroline concentration of 1 and 100 μM, n=9 for 5 μM, n=7 for 10 μM). Asterisks (*) indicate statistically significant differences between control and experiments ($p<0.05$).

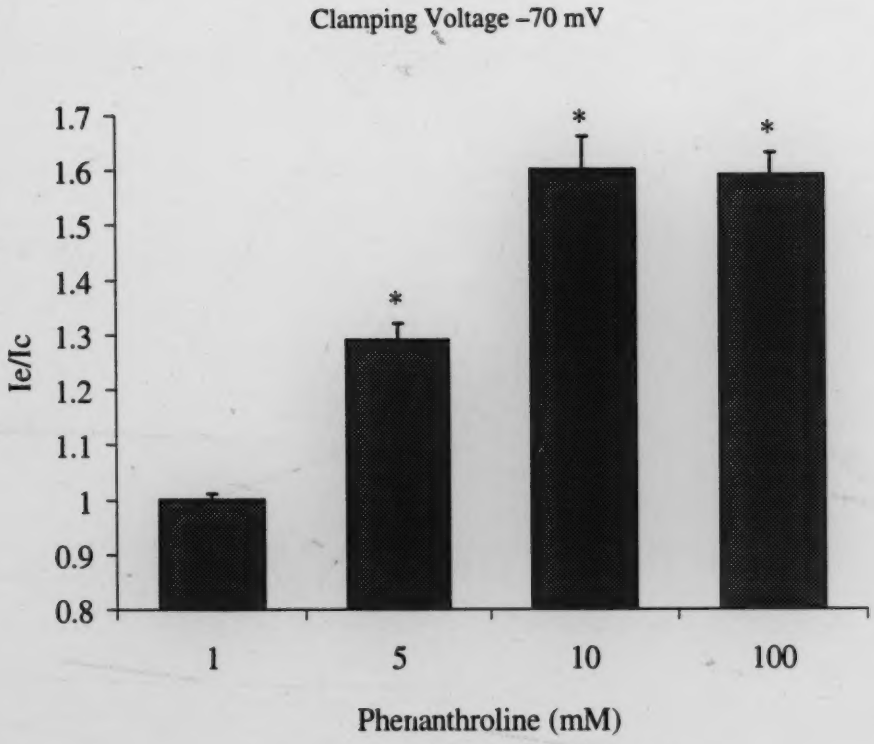

Figure 4-7. Dose-dependent stimulatory effects of 1,10-phenanthroline on whole-cell Cl^- current in Calu-3 cells. The histograms show the relative current to control at clamping voltage of -70 mV as phenanthroline concentration is increased (n= 6 for phenanthroline concentration of 1 and 100 μM, n=9 for 5 μM, n=7 for 10 μM). Asterisks (*) indicate statistically significant differences between control and experiments ($p<0.05$).

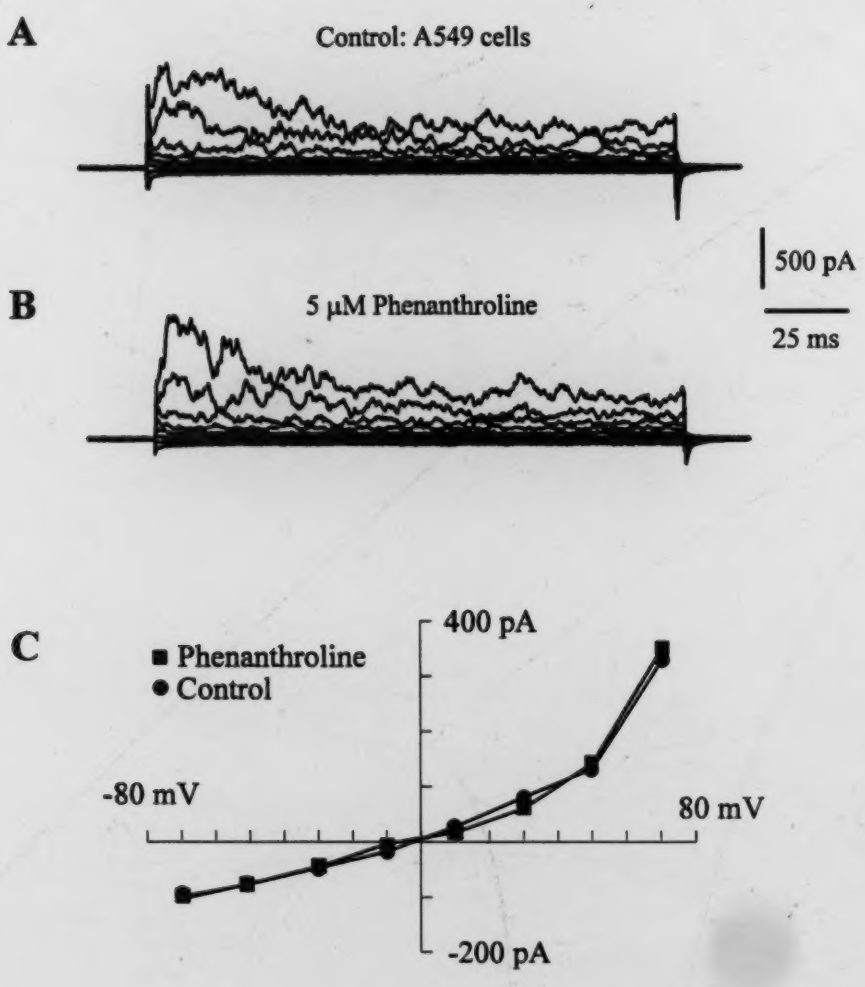

Figure 4-8. Typical recordings of whole-cell Cl^- current in A549 cells in absence (A) and presence (B) of 5 μM 1,10-phenanthroline. C). I-V relationship corresponding to recordings shown in panels (A) and (B).

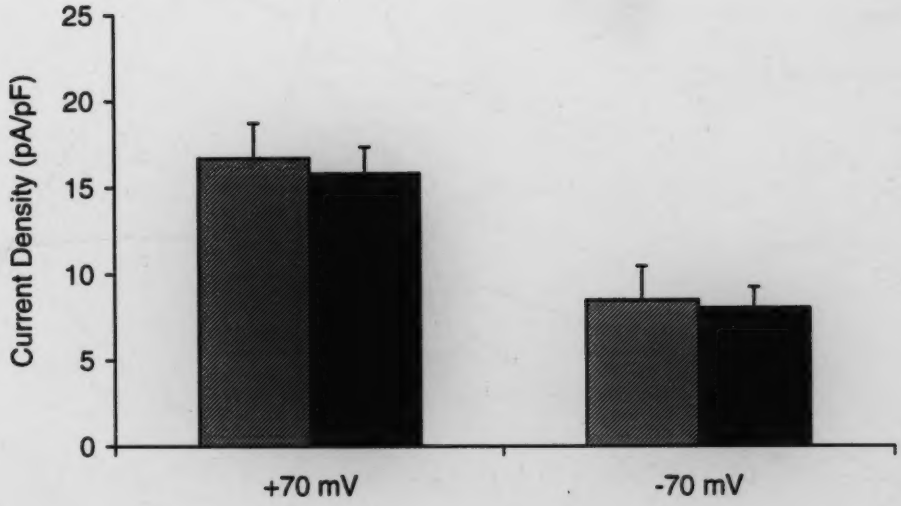

Figure 4-9. Effect of 1,10-phenanthroline on whole-cell Cl^- current in A549 cells. Shadowed bars: control; gray bars: current density after the treatment of 5 μM 1,10-phenanthroline. There is no statistically significant difference between control and phenanthroline treatment ($p>0.05$, $n=5$).

Table 4-1. Summary of the effect of phenanthroline on whole-cell Cl^- current in A549 cells.

Concentration of Phenanthroline	Current ratio (Ie/Ic) (at +70 mV)	Significance (p value)	Current ratio (Ie/Ic) (at -70 mV)	Significance (p value)
5 μM (n=5)	1.05 ± 0.04	> 0.05	1.07 ± 0.06	> 0.05
10 μM (n=6)	0.97 ± 0.08	> 0.05	1.00 ± 0.04	> 0.05

4.2.2 The effect of DPC on phenanthroline-stimulated whole-cell Cl^- currents in Calu-3 cells

To provide further evidence that the phenanthroline-affected Cl^- current in Calu-3 cells was mostly conducted by CFTR, the CFTR-specific blocker, diphenylamine-2-carboxylate (DPC), was applied to the bath solution 5 minutes after the Cl^- channels were stimulated by 10 μM 1,10-phenanthroline. Typical recordings from this experiment along with their I-V curves are shown in Figure 4-10. It is found that phenanthroline-stimulated Cl^- current was abolished by 75.86 ± 3.04% and 72.57 ± 4.21% at the clamping voltage of +70 mV and –70 mV, respectively (n=7). The residue current 5 minutes after the application of 1 mM DPC was about 30 % of basal current and about 20% of phenanthroline stimulated current at both +70 mV and –70 mV of clamping voltages (Figure 4-11). This result further indicates that the majority of whole-cell Cl^- current in Calu-3 cells is mainly mediated by CFTR Cl^- channels. The result also suggests that these channels are probably the targets of phenanthroline actions.

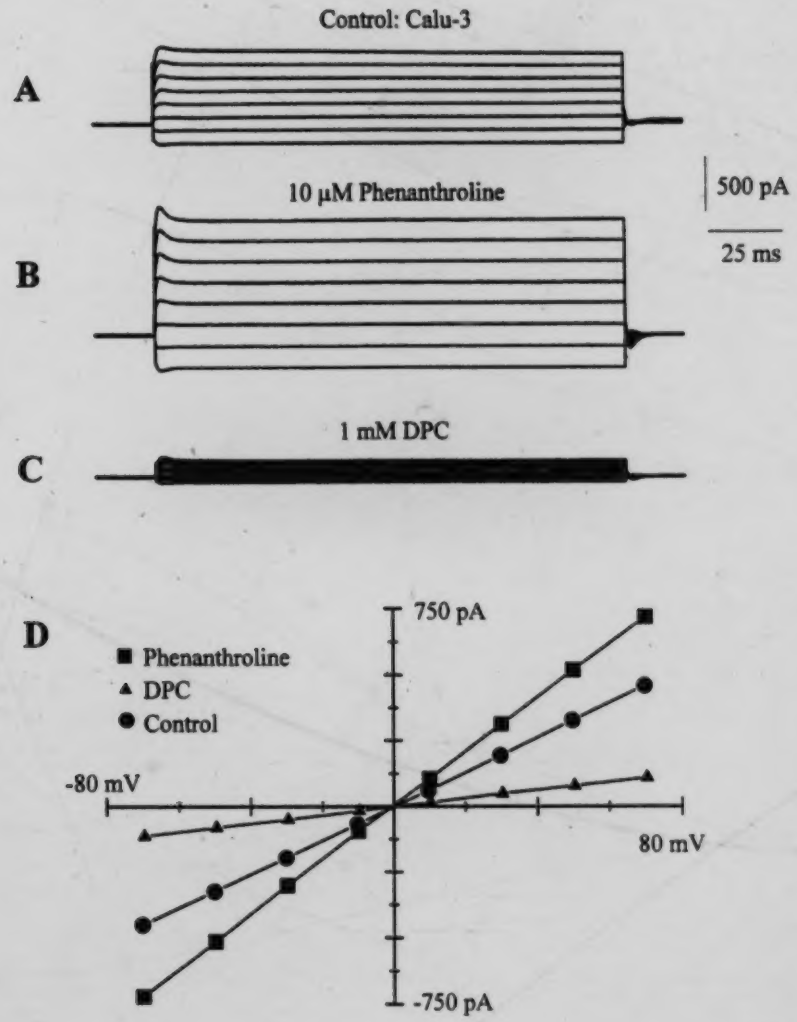

Figure 4-10. Typical recordings of whole-cell Cl^- current along with their I-V relationship showing the effect of DPC on phenanthroline stimulated Cl^- current in Calu-3 cells. Whole-cell Cl^- current was recorded at the unstimulated condition (A), after the treatment of 10 μM 1,10-phenanthroline (B), and after the addition of 1 mM DPC (C). D). I-V relationship plotted from panels A, B, and C.

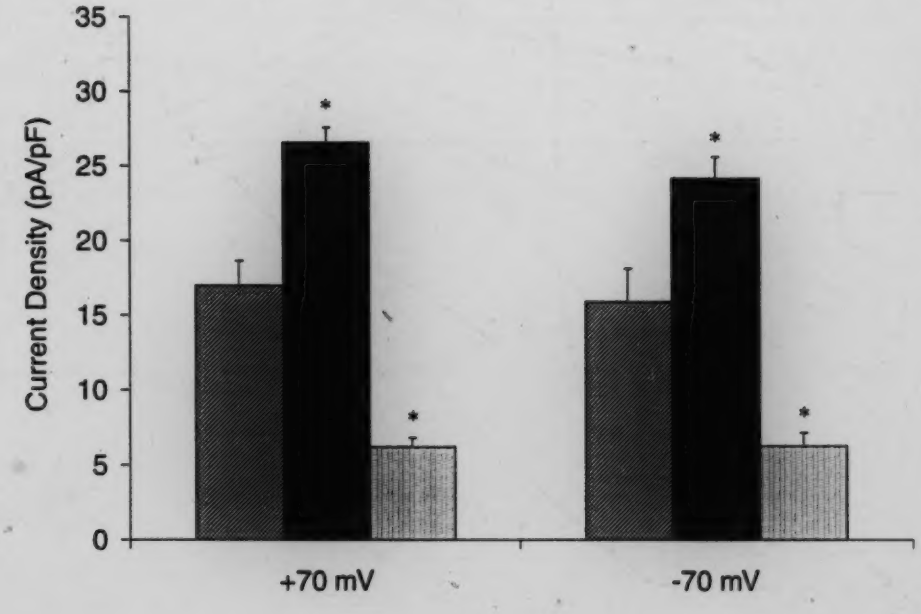

Figure 4-11. Effect of DPC on phenanthroline stimulated whole-cell Cl^- current in Calu-3 cells. Shadowed bars: control; Grey bars: current density after the treatment of 10 μM 1,10-phenanthroline; Strip bars: current density after application of 1 mM DPC (n=7). Asterisks (*) indicate statistically significant differences between control and experiments ($p<0.05$).

4.2.3 The effects of antibodies against MMP-2 and MMP-9 on the whole-cell Cl^- currents of Calu-3 cells

Since 1,10-phenanthroline is a non-specific inhibitor of MMPs, polyclonal antibodies against MMP-2 or MMP-9 were used in an attempt to provide more specific evidence for the effect of MMPs on whole-cell Cl^- current. This experiment aimed further to determine the regulatory effect of MMPs on Cl^- channels of Calu-3 cells. It also helped to decide the reaction side of MMPs with Cl^- channels since 1,10-phenanthroline is a membrane permeable chemical but antibodies do not cross the cell membrane thus can only react with Cl^- channels on the side where they are applied. For the reasons discussed earlier, this experiment restricts the MMPs under the investigation to MMP-2 and MMP-9. The antibodies were applied to the bath solution of only Calu-3 cells because the inhibitor of MMPs 1,10-phenanthroline only affected the Cl^- currents of Calu-3 cells but not A549 cells, based on the results of previous experiments.

In order to exclude any nonspecific effect of these antibodies on the Cl^- current, identical experiments were carried out using nonspecific antibodies IgG from rabbit serum as a control. The results show that 1 μg/ml of IgG did not affect Cl^- channel activities of Calu-3 cells ($n=5$, $p>0.05$).

The polyclonal antibodies against MMP-2 (1 μg/ml) applied on the extracellular side showed a significant enhancement of the whole-cell Cl^- current in Calu-3 cells. This stimulatory effect was similar to that observed with the application of 1,10-phenanthroline. At 1 μg/ml, anti-MMP-2 antibodies increased the Cl^- current by 45.57 ±

12.97 % at the clamping voltage of +70 mV. The mean normalized current density was enhanced by 7.48 ± 0.92 pA/pF, and 6.70 ± 1.14 pA/pF at the clamping voltage of +70 mV and -70 mV, respectively (n=7, $p<0.05$). Except for the increased current amplitude, the typical linear I-V relationship and time course were not altered, indicating that the current affected by anti-MMP-2 antibodies was potentially mediated by CFTR Cl^- channels. Figure 4-12 shows typical recordings of whole-cell Cl^- current before and after the application of anti-MMP-2 antibodies along with their voltage-current relationship. The normalized current densities of control and experiment at the clamping voltage of +70 mV and –70 mV were shown in Figure 4-13.

In contrast, when anti-MMP-9 antibodies were applied at the concentration of 1 µg/ml to the bath solution of Calu-3 cells, no significant effect was observed (n=5, $p>0.05$). The current ratio of experiment to control (I_e/I_c) for anti-MMP-2 and anti-MMP-9 antibody experiments at clamping voltages of +70 mV and –70 mV along with their significance (p value) are summarized in Table 4-2.

Table 4-2 Summary of the effect of antibodies against MMP-2 and MMP-9 on whole-cell Cl^- current in Calu-3 cells.

Antibodies	Current ratio (I_e/I_c) (at +70 mV)	Significance (p value)	Current ratio (I_e/I_c) (at -70 mV)	Significance (p value)
Anti-MMP2 antibodies (n = 7)	1.46 ± 0.05	<0.01	1.49 ± 0.07	<0.01
Anti-MMP9 antibodies (n = 5)	1.03 ± 0.02	>0.05	1.01 ± 0.03	>0.05

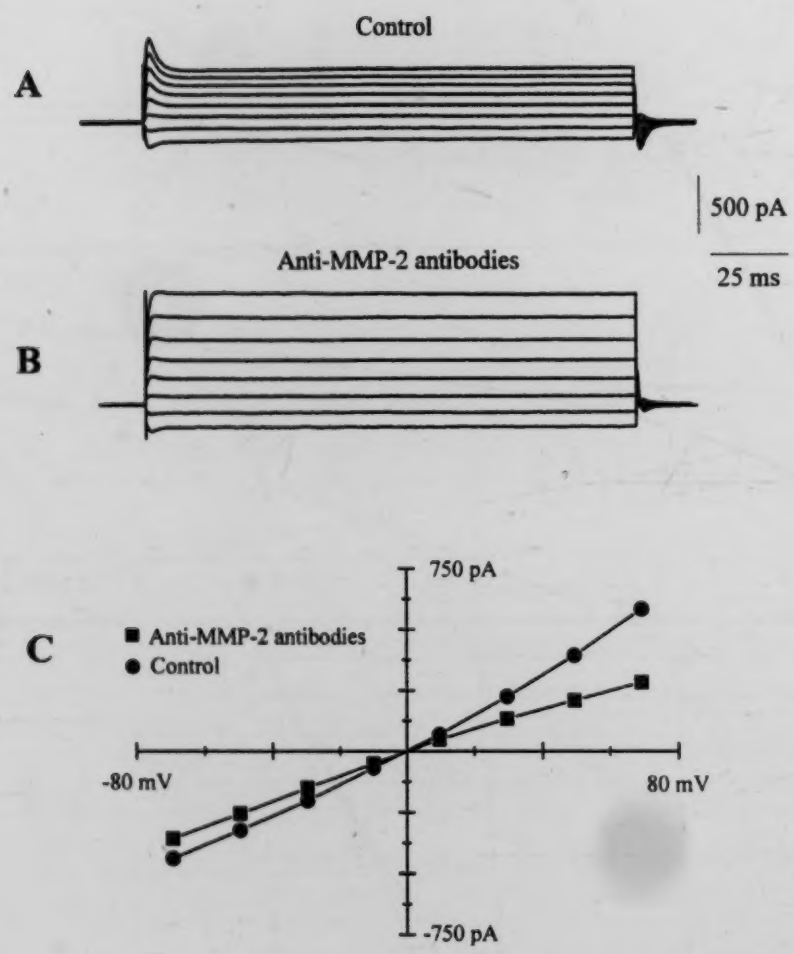

Figure 4-12. Typical recordings of whole-cell Cl- currents in Calu-3 cells in presence (A) and absence (B) of anti-MMP-2 antibodies along with their I-V relationships (C).

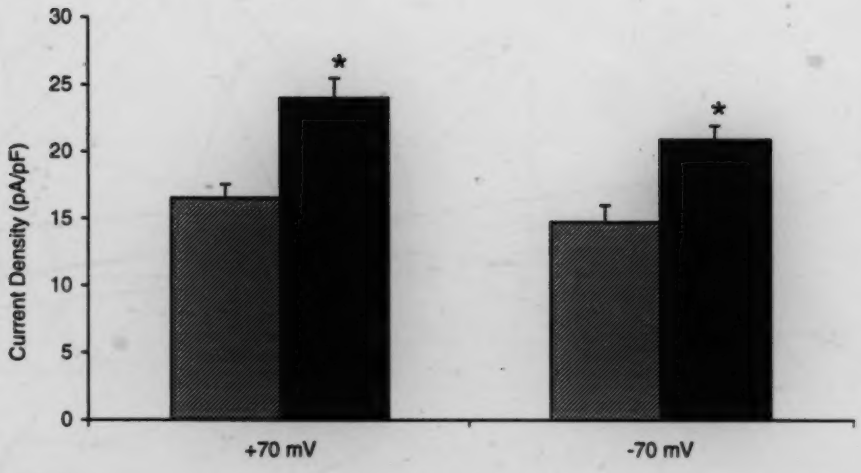

Figure 4-13. Effect of anti-MMP-2 antibodies on whole-cell Cl^- currents in Calu-3 cells. Shadowed bars: control; gray bars: current density after the application of anti-MMP-2 antibodies (n=7). Asterisks (*) indicate statistically significant differences between control and experiments ($p<0.05$).

4.2.4 The effects of human recombinant MMP-2 (rMMP-2) on the whole cell Cl^- currents in Calu-3 cells

To further investigate the regulatory role of MMP-2 on whole cell Cl^- currents, human recombinant MMP-2 (rMMP-2) activated by MMP activator p-aminophenylmercuric acetate (APMA) was applied to the bath solution at the concentrations of 0.3 ng/ml and 30 ng/ml. Considering that APMA could also activate the native MMP-2 of Calu-3 cells, similar experiment with the same APMA concentration but without rMMP-2 was also carried out as a comparison. The results showed no significant effect for APMA on the whole-cell Cl^- currents ($n=5$, $P>0.05$).

As shown in Figure 4-14, a significant inhibitory effect was observed on whole-cell Cl^- current following the application of 30 ng/ml human recombinant MMP-2. The steady state Cl^- current was decreased by $34.00 \pm 4.09\%$ and $31.25 \pm 5.96\%$ at a clamping voltage of +70 mV and -70 mV, respectively ($n=7$, $p<0.05$). The inhibitory effect on the whole-cell Cl^- current after the application of rMMP-2 was observed without changing their linear I-V behavior or reversal potential, indicating that rMMP-2 probably inhibited CFTR-mediated Cl^- current. The normalized Cl^- current density for the control and experiment at the clamping voltage of +70 mV and –70 mV were shown in Figure 4-15. In contrast, applying rMMP-2 at the concentration of 0.03 ng/ml showed no significant inhibitory effect on the whole-cell Cl^- current in Calu-3 cells ($n=3$, $p>0.05$). The result is summarized in Table 4 - 3.

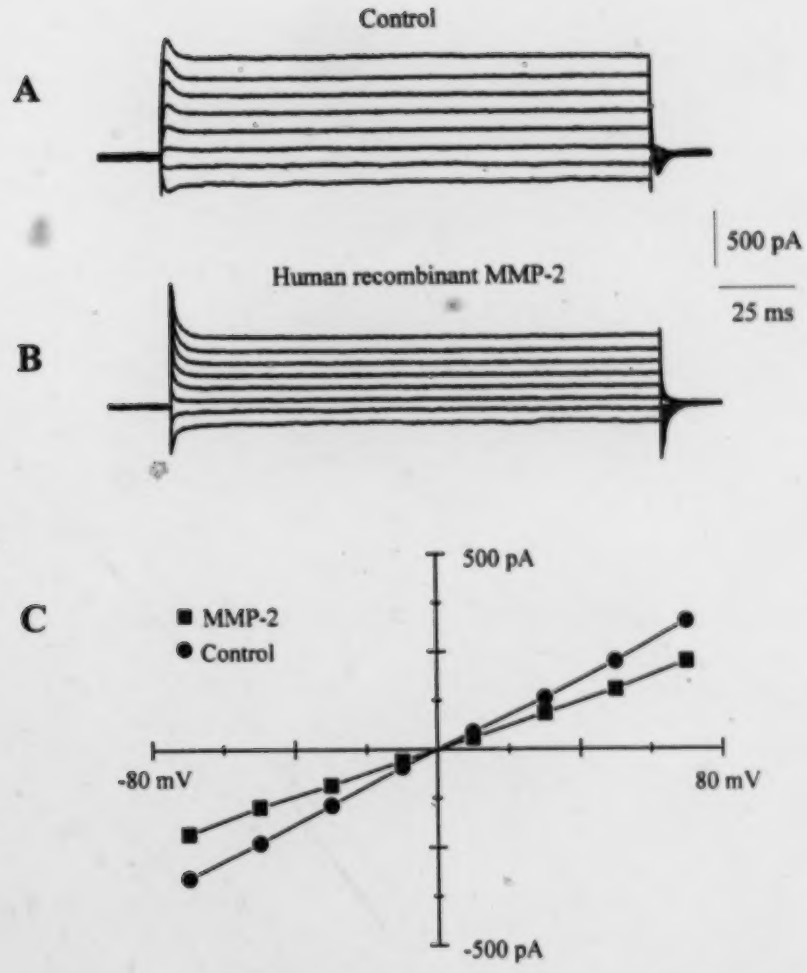

Figure 4-14. Typical recordings of whole-cell Cl^- currents in Calu-3 cells in presence (A) and absence (B) of 30 ng/ml rMMP-2 along with their I-V relationships (C).

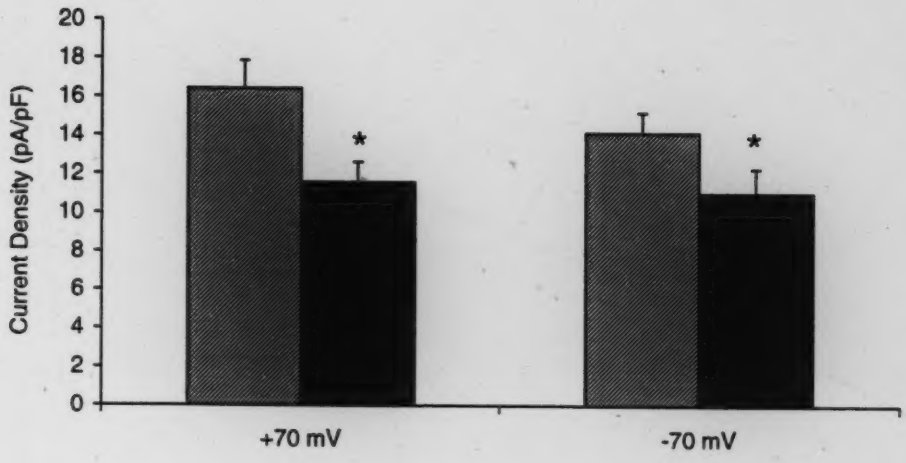

Figure 4-15. Effect of rMMP-2 on whole-cell Cl^- currents in Calu-3 cells. Shadowed bars: control; gray bars: current density after the application of rMMP-2 (30 ng/ml) (n=7). Asterisks (*) indicate statistically significant differences between control and experiments ($p<0.05$).

Table 4-3 Summary of the effect of human recombinant MMP-2 on whole-cell Cl^- current in Calu-3 cells.

Concentration of rMMP-2 (ng/ml)	Current ratio (I_e/I_c) (at +70 mV)	Significance (p value)	Current ratio (I_e/I_c) (at -70 mV)	Significance (p value)
30 (n = 7)	0.70 ± 0.05	<0.05	0.76 ± 0.08	<0.05
0.3 (n = 3)	1.05 ± 0.02	>0.05	1.05 ± 0.03	>0.05

4.3 The effect of MMPs on single-channel Cl^- current

Excised single channel patch clamp technique with inside-out configuration was applied to further identify the Cl^- channel under the regulation of MMPs. Single channels with conductance of 8.64 ± 1.21 pS (n=4) were recorded and the I-V curve shows linear current-voltage relationship. The open probability of the channels recorded was 0.53 ± 0.11 (n=4) at the basal condition and was voltage-independent. After the application of 10 μM 1,10-phenanthroline to the bath solution, the open probability of the channels was significantly increased (n=4, $p<0.05$) from 0.53 ± 0.11 to 0.91 ± 0.04 and the single channel conductance remain unchanged. Figure 4-16 shows typical recordings of a Cl^- channel activity before and after the addition of 10 μM 1,10-phenanthroline at the clamping voltage of -60 mV. The distribution of current amplitudes corresponding to this recording is shown in Figure 4-17. From the results it is evident that 1,10-phenanthroline

not only reduced time for which the channel remains in the closed state, but also induced the activation of other channels, as it is indicated by the appearance of new peaks in the distribution of current amplitudes (Figure 4-17). The current-voltage relationship corresponding to the recording shown in Figure 4-16 is plotted in Figure 4-18 and the mean open state probability for the basal condition and in the presence of 10 μM phenanthroline is shown in Figure 4-19. These results indicate that 1,10-phenanthroline affects CFTR Cl^- channel gating mechanisms but has no effect on channel pore conductive properties.

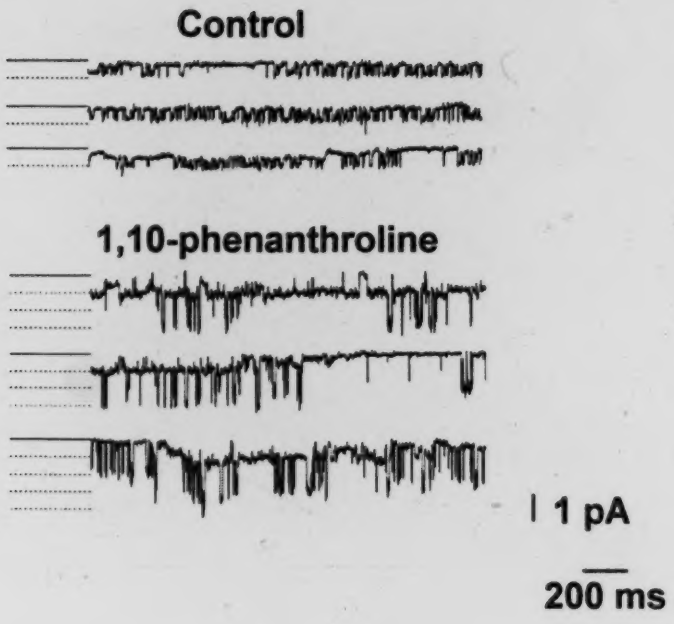

Figure 4-16. Typical recordings of single Cl^- channel event in Calu-3 cells in the presence and absence of 1,10-phenanthroline at clamping voltage of -60 mV. Dotted lines indicate open conditions in all cases. Solid lines indicate close conditions in all cases.

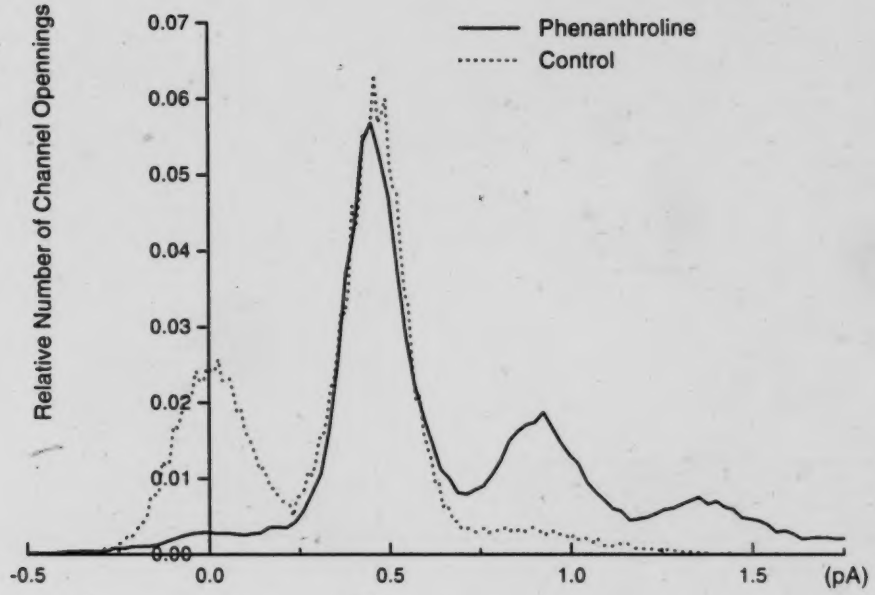

Figure 4-17. The distributions of current amplitude in the presence and absence of phenanthroline corresponding to the recordings shown in Figure 4-16.

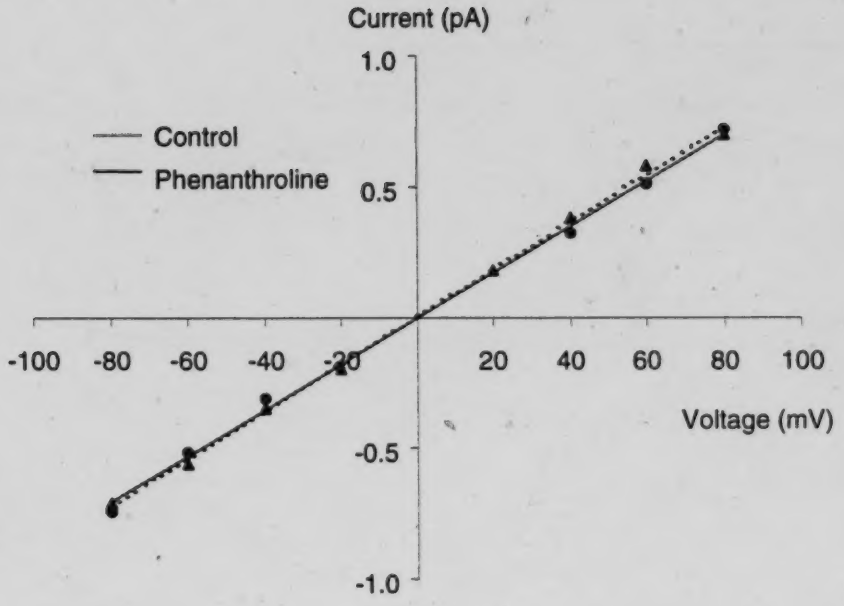

Figure 4-18. Current-voltage relationship of the recordings in the presence and absence of phenanthroline shown in Figure 4-16.

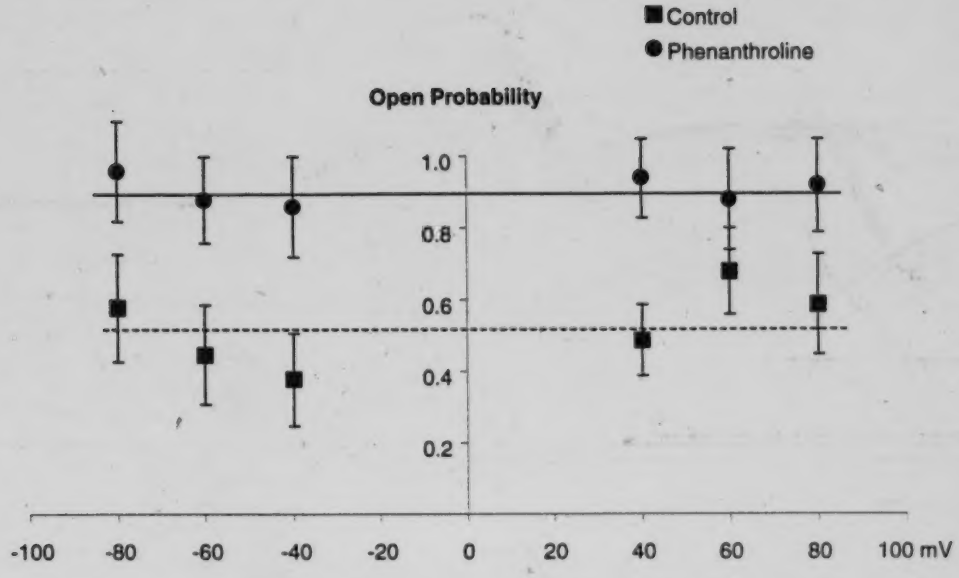

Figure 4-19. Average open probabilities calculated from four single channel recordings of 8.64 ± 1.21 pS Cl^- channel in excised inside-out patch in the absence and presence of 10 μM 1,10-phenanthroline.

Chapter 5 Discussion

This is the first report that shows a relationship between MMP-2 and whole-cell Cl^- current in human airway epithelial cells. The coupling between Cl^- movement and mucus secretion has been suggested before (Engelhardt et al. 1994). Phenanthroline has been shown to inhibit histamine-induced degranulation of serous cells (Sommerhoff et al. 1996), and some MMPs have been implicated in mucus degradation (Crowther et al., 1987). If there is a relationship between MMPs, mucus and Cl^- secretion, it remains to be established. However, it is tempting to hypothesize that the effect of MMPs may be especially important under pathological conditions, since increased expression of MMPs in inflammation will contribute to mucus degradation, while inhibition of CFTR will cause its dehydration.

5.1 The significance of MMP presence in human airway epithelial cells

Matrix metalloproteinases (MMPs) are thought to be the major physiologically relevant mediators of extracellular matrix degradation (Ries & Petrides, 1995). They can cleave most of the protein constituents of the extracellular matrix, facilitating cell movement, tissue remodeling and repair. The main source of MMPs in the lung are stromal cells including fibroblasts, endothelial cells, osteoblasts, keratinocytes and chrondrocytes (O'Connor & FitzGerald, 1994). They play a role in normal lung development and the maintenance of the extracellular matrix scaffold, which supports the

alveolar structure and is essential for normal lung functions. Recently, increasing reports showed that airway epithelial cells secrete MMP-2 or/and MMP-9. The possibility that airway epithelial cells produce these MMPs was first suggested by Niles et al when they made the observation that these cells in culture progressively degrade collagen substrates under certain growth conditions (Niles et al., 1988). Several subsequent studies provided direct evidence. Collier et al. (1988) first reported that Ha-ras oncogene-transformed human bronchial epithelial cells secreted a 72 KDa gelatinase (MMP-2 or gelatinase A). Recently, a study reported that 92 KDa gelatinase (MMP-9 or gelatinase B) mRNA was detected in normal pulmonary tissue and bronchial epithelium by in situ hybridization (Canete-Soler et al., 1994). Another study showed the expression of 72 KDa gelatinase (MMP-2) in bovine tracheal gland serous cells (Tournier et al., 1994). This MMP-2 was found to locate at the periphery of some tracheal gland acini and to be involved in gland development. Finally, the primary cultures of human bronchial epithelial cells were known to express 92 KDa gelatinases (MMP-9) constitutively by immunological, enzymatic and RT-PCR data (Yao et al., 1996).

The results of this research clearly show that A549 and Calu-3 cells derived from human airway surface epithelia and submucosal gland serous cells, respectively, produce two gelatinolytic enzymes, MMP-2 and MMP-9. The following evidence indicates that these enzymes are the members of the matrix metalloproteinase family: 1). They are detected as a major band at 72 KDa corresponding to the proenzyme form of MMP-2 and a minor band at 92 KDa corresponding to the proenzyme form of MMP-9; 2). They have gelatinolytic activities; 3). They are Zn-dependent: the metal chelator such as 1,10-phenanthroline inhibited their activities; 4). Specific anti-MMP-2 and anti-MMP-9

antibodies recognized them. These results are consistent with the previous reports that airway epithelial cells produce and release gelatinolytic matrix metalloproteinases.

Although Calu-3 and A549 cells represent different models of airway epithelial cells (see Chapter 2), the profile of MMPs secreted by these two cell lines are very similar (large amounts of MMP-2 and small amounts of MMP-9). This result suggests that MMP-2 and MMP-9 expressed by Calu-3 and A549 cells may play a role in extracellular matrix remodeling and tracheobronchial gland morphogenesis. Meanwhile, MMP-2 and MMP-9 might be involved in the interaction between MMPs and Cl^- channel proteins.

5.2 The recorded whole-cell current in Calu-3 cells is dominated by CFTR Cl^- channels

Secretory processes in airway submucosal gland epithelial cells contribute to homeostasis and to the first-line defense against inhaled insult (Fung & Rogers, 1997). The submucosal gland serous cells, rather than the mucous cells, appear to be the cells primarily involved in providing the liquid component of gland secretions. Immunocytochemical studies have shown the highest concentration of CFTR in human airways is on the serous cells of the submucosal glands (Engelhardt et al., 1992). This indicates that CFTR Cl^- channels in submucosal gland serous cells are the major component of Cl^- transport responsible for the normal ASL composition and quantity and thus efficient mucociliary clearance. The evidence that highest level of CFTR is

expressed in airway submucosal gland serous cells implicates them in CF lung disease (Engelhardt et al., 1992).

Calu-3 cells were derived from a human lung adenocarcinoma. Several studies suggest that they may be a useful model for study of submucosal gland cells. These cells have a serous phenotype on the basis of several important enzyme markers (Finkbeiner et al, 1993), and more importantly, they express the highest levels of CFTR (Finkbeiner et al, 1993; Shen et al., 1994). Functionally, Calu-3 cells form a polarized epithelium with tight junctions. They show a cAMP-dependent Cl^- secretion and the Cl^- conductance was mainly mediated by CFTR Cl^- channels (Shen et al., 1994; Haws et al., 1994).

It is well established that the CF gene products, CFTR, is a cAMP-activated Cl^- channel, which provides a route for Cl^- exit across the apical membrane of Cl^- secreting mammalian epithelia (Anderson et al., 1991a; Tabcharani 1991; Bear et al., 1992). They are 8-10 pS in single-channel conductance, linear in current-voltage relationship, independent on voltage and time, and sensitive to DPC blockade but insensitive to stilbene such as DIDS. These distinct biophysical properties distinguish CFTR from other types of Cl^- channels.

The experiment was designed to record the whole-cell Cl^- current, even though the cell membrane of Calu-3 cells contains several different groups of ion channels, especially Na^+, K^+, and Cl^- channels, which are thought to be the major determinants of membrane potential. To achieve this, bath-to-pipette ion gradients that fix the equilibrium potentials for these ions at different values were used. To simulate physiological conditions, the pipette contained high KCl (140 mM) and bath high NaCl (140 mM). Under this condition, the equilibrium potentials for Na^+, K^+, and Cl^- ions were about 85, -

85, and 0 mV, respectively. The results showed that under this non-symmetrical ionic conditions the reversal potential of the current was –0.4 ± 5.2 mV, suggesting that at resting conditions there are far more open Cl^- selective channels than Na^+ or K^+ selective channels. This conclusion was further supported by the blockade of the current with CFTR blocker DPC.

As described in Chapter 1, there are at least four groups of Cl^- channels located on the apical membrane of airway epithelia, and different types of airway epithelia have different distribution of these Cl^- channels. For example, airway submucosal gland serous cells have the highest level of expression of CFTR, while airway surface epithelia are dominated by non-CFTR Cl^- channels. The following discussion will show that the current recorded in Calu-3 cells was mainly mediated by CFTR Cl^- channels.

Since there is no previous reports on the whole-cell Cl^- current in Calu-3 cells, direct comparison is not available. However, the whole-cell patch clamp recordings obtained from Calu-3 cells here have a similar pattern and time course to the recordings in other epithelial cells which are rich in CFTR or cells that express the recombinant CFTR (Naren et al., 1997). The voltage-independence and the linear I-V relationship with a chloride-selective reversal potential indicate that CFTR Cl^- channels in Calu-3 cells mediated the whole-cell Cl^- current. The channel blockade experiment provides further evidence. It shows that more than 70% of the Cl^- current stimulated by 1,10-phenanthroline was abolished by CFTR blocker DPC.

Single channel patch clamp studies showed that channels with conductance of about 8.64 pS were repeatedly recorded at both basal and stimulated conditions. The channels have linear I-V relationship with the voltage-independent open state probabilities. These

observed biophysical properties of the channel studied are in agreement with those of endogenously expressed or recombinant CFTR (Haws et al., 1994).

Under resting conditions in many cells, CFTR-dependent apical membrane Cl^- conductance is nearly zero (Hanrahan et al., 1993). In contrast, basal Cl^- current was repeatedly observed in whole-cell patch clamp study in this work. This basal Cl^- current shows a linear I-V relationship and voltage-independence with Cl^--selective reversal voltage. The residue current after DPC blockade was about 30% of the basal current. In the single channel patch clamp study, the average open probability of recorded Cl^- channels (at about 8.64 pS) was 0.53 ± 0.11 with linear I-V relationship and voltage-independence. These results strongly suggest that part of CFTR Cl^- channels may be constitutively activated under resting conditions in Calu-3 cells. They are in total agreement with two recent reports obtained with Calu-3 cells. One of the reports was from an Ussing chamber study showing a baseline oscillation in short circuit current (I_{sc}), which can be increased by cAMP stimulators and dampened or abolished by DPC (Shen et al., 1994). Another from a single-channel study showing that, at room temperature, active CFTR channels in more than 50% of cell-attached patches of unstimulated Calu-3 cell apical membranes were present (Haws et al., 1994).

In summary, the results of this work, both from whole-cell and single-channel patch clamp studies showed that Calu-3 cells express high level of CFTR that dominates the Cl^- conductance in the apical membrane of these cells. The evidence includes 1). Cl^--selective reversal potential, i.e. for symmetric Cl^- solution in both extracellular and intracellular sides, the reversal potential for Cl^- current is at zero; 2). Linear I-V relationship observed in both whole-cell and single-channel patch clamp methods; 3).

Most whole-cell Cl^- current was dampened by DPC but not DIDS; 4). Voltage-independence; 5). Single channel conductance of about 8.64 pS which was consistent with previous reports.

In contrast to Calu-3 cells, strong outwardly rectifying whole-cell current was observed in A549 cells. This result indicated that A549 cells, a model for the study of airway surface epithelia, conducted Cl^- current which was mediated mostly by non-CFTR Cl^- channels, and is consistent with previous observations (Renier et al., 1995). Moreover, the treatment with MMP inhibitor, 1,10-phenanthroline had no effect on either the amplitude or time course of the whole-cell Cl^- current of these cells. This result suggests that MMPs have no effect on ion movement in airway surface epithelial cells in which non-CFTR Cl^- channels are predominant.

5.3 The role of MMPs in regulation of ion movement

In addition to traditional regulatory mechanisms of epithelial ion channels, novel regulatory pathways have recently been discovered. It was observed that luminal factors, such as urinary proteases, kallikrein and urokinase, had a direct inhibitory effect on epithelial sodium channel (Lewis & Clausen, 1991). Another paper reported that the amiloride-sensitive sodium channel was activated by an epithelial serine protease (Vallet et al., 1997). Moreover, it was demonstrated that an epithelially expressed syntaxin, a membrane protein that also modulates neurosecretion and Ca^{2+} channel gating in brain, down regulated CFTR-mediated Cl^- current probably through protein-protein interaction (Naren et al., 1997).

On the other hand, while it is well established that MMPs play a key role in the extracellular matrix degradation, novel functions of MMPs were being uncovered. In the lung, MMPs were shown to affect the viscoelastic properties of mucus (Wesley et al., 1983) and degranulation of human submucosal gland cells (Sommerhoff et al., 1996). Since submucosal glands are the major expression sites of CFTR Cl^- channels, which are involved in mucus secretion, it is possible that MMPs could affect mucociliary clearance by regulating CFTR activity. Under this circumstance, the possible interaction between MMPs and epithelial Cl^- channels was explored in this work for the first time.

Although both Calu-3 and A549 cells secret MMP-2 and MMP-9, the dose-dependent stimulation of phenanthroline was only observed on the whole-cell Cl^- current in Calu-3 cells. Since in Calu-3 cells the whole-cell Cl^- current is mainly mediated by CFTR, while in A549 cells by non-CFTR Cl^- channels, this result indicates that MMPs might exert an inhibitory effect on the activity of CFTR Cl^- channels but not that of non-CFTR Cl^- channels. Because phenanthroline inhibits MMP activity by chelating Zn^{2+} which functions as a cofactor or structural component of MMPs as well as many other enzymes and some ion channels (Harrison & Gibbons, 1994), it is questioned if the observed stimulatory effect of phenanthroline on CFTR Cl^- current is attributed to MMPs. If it is, which MMP member is involved and what is the mechanism of the interaction? Does the interaction take place on the cytosolic side or extracellular side, since phenanthroline is a membrane permeable chemical?

The results have shown that Calu-3 cells express large amounts of MMP-2 and small amounts of MMP-9, therefore polyclonal antibodies against MMP-2 and MMP-9 were used to investigate if these gelatinases were involved in the interaction of MMPs

and CFTR Cl^- channels. Anti-MMP-2 antibodies were applied to the bath solution to neutralize the activity of endogenously expressed MMP-2. It was found that this resulted in a similar potentiation of whole cell Cl^- current to that observed in phenanthroline treatment in Calu-3 cells. Different results were obtained with anti-MMP-9 antibodies that did not alter the whole cell Cl^- current in Calu-3 cells. This suggests that MMP-2 probably participate in the regulation of Cl^- channels on the extracellular side since anti-MMP-2 antibodies are cell membrane impermeable molecules. The augmentation of whole-cell Cl^- current by phenanthroline and anti-MMP-2 antibodies suggests that under the basal conditions, probably CFTR Cl^- current have already been tonically inhibited by endogenously expressed MMP-2.

The direct evidence comes from the inhibitory effect observed after the treatment of Calu-3 cells by addition of activated human recombinant MMP-2 in extracellular side. However, further study has to be done in order to elucidate the mechanism underlying the interaction between MMP-2 and CFTR Cl^- channels. The regulation of CFTR Cl^- channels could occur through the cleavage of the channel proteins by MMP-2, or through the physical interaction by the direct binding of MMP-2 and CFTR proteins. MMP-2 could decrease CFTR mediated Cl^- current by modifying the channel pore, thereby changing the single-channel conductance and ionic selectivity; or modifying the gating kinetics and thus decrease the open probability of the channel. The single-channel patch clamp results presented here showed that inhibition of MMP activity by phenanthroline only increased the open probability of CFTR channels and did not modify single channel conductance. This indicated that the gating kinetics was affected but the channel pore

properties were not. Further evidence should be obtained by directly applying the human recombinant MMP-2 in the single-channel patch clamp study.

All experiment recordings in this work showed that, after the addition of 1,10-phenanthroline, anti-MMP-2 antibodies, or human recombinant MMP-2, only the amplitude of the whole-cell Cl^- current was alternated, while the patterns, time courses, and I-V relationship of current recordings were not. This provided further evidence that Cl^- currents regulated by MMP-2 were mediated by CFTR Cl^- channels.

MMP-2, like all other MMPs, is produced in a proenzyme form, which is enzymatically inactive (O'Connor & FitzGerald, 1994). However, MMP-2 is unique in that it is not activated by serine proteinases such as plasmin, plasma kalikrein, neutral elastase or cathepsin G, which are considered physiological activators of MMP family. Instead, MMP-2 is specifically activated on the cell surface by members of a new subfamily of the MMPs – the membrane type MMPs (MT-MMPs) (Sato & Seiki, 1996; Butler et al., 1997). In addition to this unique mechanism of activation, MMP-2 was also known to be anchored on the cell membrane by specifically binding to certain membrane-bound molecules. MT1-MMP has been associated with the activation of MMP-2 as well as binding MMP-2 molecules on cell surface in different cells (Sato et al., 1996; Okada et al., 1997). It was reported that TIMP-2 is capable of localizing MMP-2 to the surface of HT-1080 cells via interaction with a specific binding of cell membrane associated protein (Emmert-Buck et al., 1995). Another study showed that MMP-2 was localized to the invasive cell surface by interaction with cell surface adhesive molecule, integrin $\alpha v\beta 3$ (Brooks et al, 1996).

It has been found in this work that TIMP-2 was also present in Calu-3 cells and may play a role in the regulation of MMP-2 activity and locating MMP-2 on the cell surface. MMP-2 may also be activated by MT-MMPs located on the cell surface and anchored by them or other cell membrane associated molecules such as integrins. This could be a reason why MMP-2, which is generally thought to be secreted into the medium and washed out before the patch clamp study, can still exert an inhibitory effect on the activity of CFTR Cl^- channels.

The result in this study also suggests that the endogenous MMP-2 located on the extracellular cell surface might have already been activated and interacted with CFTR proteins. This was supported by two facts: 1). Application of anti-MMP-2 antibodies to neutralize endogenously expressed MMP-2 increased whole-cell Cl^- current, and 2). Application of MMP activator APMA into the bath solution did not produce further inhibition on Cl^- currents (see 4.3). This observation also indicates that, at the basal condition, MMP-2 has already tonically down regulated the CFTR Cl^- channel activities. On the other hand, the application of exogenous activated MMP-2 exhibited a further decrease of Cl^- current on the basic regulation of endogenous MMP-2, suggesting that there is probably some space for the further regulation of CFTR mediated Cl^- current under physiological and pathological conditions.

5.4 The mechanisms of CFTR - MMP-2 interactions in human airways: an hypothesis

Presently, there are several reports linking ion channel function to endogenous extracellular proteins. It was shown that the activity of the amiloride-sensitive Na^+ channel in epithelial cells is regulated by a serine protease expressed at the surface of the same cell (Vallet et al., 1997). The mechanism of this autocrine regulation does not involve activation of a G-protein-coupled receptor, but rather proteolysis of a protein that is either a constitutive part of the channel itself or closely associated with it (Chraibi et al., 1998). In another study it was shown that CFTR interacts physically and functionally with an epithelial syntaxin 1A isoform that is a negative modulator of CFTR-mediated Cl^- currents (Naren et al., 1997). While the mechanism of these interactions is still unknown, it was suggested that this might involve a direct regulation of CFTR activity through protein-protein interactions. Similar mechanisms are expected to be involved in CFTR – MMP-2 interactions.

Base on the experiment results and previous discussions, it is hypothesized that MMP-2 secreted by calu-3 cells is anchored and activated on the extracellular cell surface, probably by MT-MMPs or TIMP-2. Through protein-protein interactions, i.e. probably by directly binding to CFTR molecules and / or cleaving the channel proteins, MMP-2 modulates CFTR-mediated Cl^- current by affecting the channel gating mechanism. It is also proposed that the CFTR-MMP-2 interactions may play a role in fine-tuning CFTR activity in response to certain physiological or pathological cues such as the activation of second messenger pathways or cytokines in the inflammatory

condition that regulate the MMP expression, secretion and activation. Understanding the mechanisms by which MMP-2 regulates CFTR activity may be relevant to the design of strategies that regulate Cl^- secretion in human airways.

Chapter 6 Conclusion

Chloride channels located in apical membrane of airway epithelial cells represent a major component of transepithelial Cl^- transport. They are crucial in the regulation of volume and composition of airway surface liquid thus important in mucociliary clearance. The defective cAMP-regulated Cl^- conductance in cystic fibrosis causes many clinical problems especially the deterioration of pulmonary function. Better understanding of this Cl^- channel gating will shed light on the treatment of cystic fibrosis. The main purpose of this thesis was to study the possible regulatory effect of matrix metalloproteinase on CFTR Cl^- channels of airway epithelia and the potential use of some drugs in CF treatment by interrupting the interaction between MMPs and CFTR.

The two human airway epithelial cell lines, Calu-3 and A549, were used as models of submucosal gland serous cells and airway surface epithelia respectively. The patch clamp studies showed that Cl^- currents conducted in Calu-3 cells were mainly mediated by CFTR Cl^- channels, whereas in A549 cells were mainly mediated by non-CFTR Cl^- channels. These results confirmed that submucosal gland serous cells express a high level of CFTR proteins while airway surface epithelia express a high level of non-CFTR Cl^- channels and provide further evidence that Calu-3 and A549 cells are very useful models in the study of airway Cl^- channels and liquid secretion.

Gelatin zymography and Western blot analysis showed that both Calu-3 and A549 cells produce and secret large amounts of proenzyme form of MMP-2 and small amounts of proenzyme form of MMP-9. This result suggests that MMP-2 and MMP-9 secreted by

Calu-3 and A549 cells may play a role in extracellular matrix remodeling and tracheobronchial gland morphogenesis. Meanwhile, they might be involved in the interaction between MMPs and Cl^- channel proteins. It was also shown that only Calu-3 cells produce detectable tissue inhibitor of metalloproteinase-2 (TIMP-2), the endogenous MMP inhibitor. This suggests that TIMP-2 might play a role in the interaction between MMP-2 and CFTR proteins.

Although both cell lines produce and secrete MMP-2 and MMP-9, patch clamp studies showed that MMP inhibitor 1,10-phenanthroline affected the whole-cell Cl^- current of Calu-3 cells but not that of A549 cells, indicating that MMPs may only affect the ion movement in submucosal gland cells. Since Calu-3 cells conducted mostly CFTR-mediated Cl^- current, it is likely that CFTR Cl^- channels were affected by MMPs. This is supported by the evidence that whole-cell Cl^- current was abolished more than 70% by the CFTR blocker, DPC. Single channel patch clamp studies on Calu-3 cells further confirm the observation that the channels affected by phenanthroline have the similar biophysical properties of CFTR Cl^- channels.

Studies using antibodies against MMP-2 or MMP-9 showed that only MMP-2 was involved in the interaction between MMPs and CFTR Cl^- channels. An inhibitory effect on whole-cell Cl^- current was observed by direct addition of human recombinant MMP-2 in the extracellular side and this confirms the previous proposal that there is an inhibitory effect of MMP-2 on CFTR Cl^- channels.

Of all MMP members, MMP-2 is special in several ways. First, it is activated in a different way from other MMP members. Second, unlike other MMPs, MMP-2 can be secreted out of the cells but anchored on the extracellular cell surface, suggesting that

MMP-2 may affect the activity of membrane associated proteins such as CFTR. Third, many functions of MMPs unrelated to the extracellular matrix degradation were attributed to MMP-2. It is hypothesized here that there may exist an interaction between MMP-2 and CFTR Cl^- channels in airway submucosal gland serous cells. The CFTR-MMP-2 interaction may play a role in fine-tuning CFTR activity and therefore contributes to the regulation of submucosal mucus secretion and mucociliary clearance. Understanding the mechanisms by which these molecules regulate CFTR activity may be relevant to the design of strategies for augmenting epithelial CFTR function in cystic fibrosis.

Future studies are needed to confirm this hypothesis and to elucidate the mechanisms underlying the MMP-2 and CFTR interaction. More single channel studies by testing the possible effects of anti-MMP-2 antibodies and rMMP-2 on single Cl^- channel activity will provide more evidence of the interactions between MMP-2 and CFTR. Direct binding of MMP-2 and CFTR in vitro could be tested by immunological methods (Naren et al., 1997). Furthermore, the interaction of these two proteins could be examined by incorporating these two molecules into lipid bilayers (Duszyk et al., 1993) or expressing the cDNAs in the cells, which do not endogenously express them (Kunzelmann et al., 1997). The possibility of mediators in the interaction of MMP-2 and CFTR could also be studied by adding the potential molecules and test the channel activities.

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